Calf Intestinal Alkaline Phosphatase, a Novel Therapeutic Drug for Lipopolysaccharide (LPS)-Mediated Diseases, Attenuates LPS Toxicity in Mice and Piglets

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ABSTRACT

It has been demonstrated that human placental alkaline phosphatase (HPLAP) attenuates the lipopolysaccharide (LPS)-mediated inflammatory response, likely through dephosphorylation of the lipid A moiety of LPS. In this study, it is demonstrated that also alkaline phosphatase derived from calf intestine (CIAP) is able to detoxify LPS. In mice administered CIAP, 80% of the animals survived a lethal *Escherichia coli* infection. In piglets, previous to LPS detoxification, the pharmacokinetic behavior of CIAP was studied. CIAP clearance was shown to be dose-independent and showed a biphasic pattern with an initial $t_{1/2}$ of 3 to 5 min and a second phase $t_{1/2}$ of 2 to 3 h. Although CIAP is cleared much faster than HPLAP, it attenuates LPS-mediated effects on hematoma and tumor necrosis factor-$\alpha$ responses at doses up to 10 $\mu$g/kg in piglets. LPS-induced hematological changes were antagonized, and the tumor necrosis factor-$\alpha$ response was reduced up to 98%. Daily i.v. bolus administration of 4000 units CIAP, the highest dose used in the LPS intervention studies, in piglets for 28 days was tolerated without any sign of toxicity. Therefore, CIAP potentially encompasses a novel therapeutic agent in the treatment of LPS-mediated diseases. Based on the data mentioned above, human clinical trials have been initiated.

Lipopolysaccharide (LPS)-mediated diseases comprise a variety of acute and chronic diseases, for example sepsis (Vincent et al., 2002) and ulcerative colitis (Obana et al., 2002). LPS is a constituent of the outer membrane of Gram negative bacteria and is essential for bacterial growth and survival (Rietschel and Wagner, 1996). It is a complex, negatively charged molecule composed of a polysaccharide chain called the O-specific chain and a lipid moiety referred to as lipid A. The latter is the actual toxic moiety of the LPS molecule and contains phosphate groups shown to be essential for its immunostimulatory activity. LPS binds to many macromolecules such as albumin, low-density lipoprotein, high-density lipoprotein, and LPS-binding protein (LBP) (Schumann, 1998; de Haas et al., 2000). When bound to LBP, the LBP-LPS complex is transferred to membrane-bound or soluble CD14, thereby enabling interactions with Toll-like receptors (TLRs) on cell membranes (Triantafilou and Triantafilou, 2002). The physiological function of signaling through TLRs is to induce the production and release of proinflammatory cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin (IL)-1, IL-6, IL-8, and IL-12 (Medzhitov, 2001; Barton and Medzhitov, 2002). Also, induction of nitric oxide, prostaglandins, leukotrienes, and toxic oxygen radicals (Lin and Lowry, 1998; Howe, 2000) is reported.

One of the most life-threatening LPS-mediated diseases, Gram negative sepsis, is characterized by excessive production of the above-mentioned proinflammatory cytokines, activation of proteolytic cascades, coagulation abnormalities (Dickneite and Leithauser, 1999), and hemodynamic responses, resulting in hypotension, poor tissue perfusion, and multiorgan failure (Lin and Lowry, 1998; Howe, 2000).

Because tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin (IL)-1 seem to be the earliest mediators of the subsequent host response (Baumann and Gauldie, 1994; Lin and Lowry, 1998; Howe, 2000), many antisepsis therapies focused on neutralizing or antagonizing these two cytokines (van der Poll, 2001). Other therapies aimed at LPS neutralization by using anti-LPS antibodies or LPS “binding” proteins, scavenging by high-density lipoprotein particles (Vesy et al., 2000), inhibiting nitric oxide synthesis, or using anticoagulants (Hoeve, 2000; van der Poll, 2001). Despite all innova-

ABBREVIATIONS: LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TLR, toll-like receptor; TNF-$\alpha$, tumor necrosis factor-$\alpha$; IL, interleukin; AP, alkaline phosphatase; CIAP, calf intestinal alkaline phosphatase; U, glycine alkaline phosphatase unit; WBC, white blood cell; AUC, area under the curve; ASGPR, asialoglycoprotein receptor.
tions in the technical management of critically ill patients and the efforts made in developing and exploring new therapeutics, sepsis still continues to be one of the leading causes of mortality and morbidity in hospitals with mortality rates of 35 to 50% (Vincent et al., 2002). Recently, the anticoagulant and anti-inflammatory agent activated protein C is reported to reduce overall mortality in severe sepsis patients with 6% (Bernard et al., 2001).

Poelstra et al. (1997b) and Bentala et al. (2002) showed that placental AP reduces mortality in mice lethally infected with Gram negative bacteria. Furthermore, this group has shown that human placental AP dephosphorylates and thereby detoxifies LPS at physiological pH levels (Verweij, personal communication). AP catalyzes the hydrolysis of phosphomonoesters with release of inorganic phosphate at alkaline pH in vitro (McComb et al., 1979). In mammals, the AP family consists of several isoenzymes classified in tissue-nonspecific APs (liver-bone-kidney type) and tissue-specific APs (intestinal, placental, and germ cell type) (Fishman, 1990).

Bovine (calf) intestinal mucosa represents a rich source of intestinal alkaline phosphatase (CIAP). Whether this AP also exerts the proposed physiological role in vivo is subject of this study. First, the data obtained with human placental AP (Bentala et al., 2002) were confirmed using CIAP in a mouse model. Here, 80% of the CIAP-treated mice survived a lethal Gram negative bacterial infection, likely due to reducing the LPS-mediated inflammatory sepsis cascade. The LPS-detoxifying capacity of CIAP was studied in a piglet model. Furthermore, piglets were treated with LPS ranging from 10 ng to 10 μg/kg body weight with or without CIAP, and inflammation parameters such as TNFα release and hematological parameters were studied. To assess the applicability of CIAP as a therapeutic agent, pharmacokinetic and safety studies were performed.

Materials and Methods

Chemicals

CIAP was obtained from Biozyme (Blaenavon, UK); *Escherichia coli* O111:B4 LPS, glycine, and 4-p-nitrophenylphosphate were from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands); MgCl₂ and NaOH were from Merck Eurolab BV (Amsterdam, The Netherlands); and heparin (5000 IE/ml) was from Leo Pharma BV (Weesp, The Netherlands).

Animals and Experimental Design

**Mice Experiments.** Specific pathogen-free female Balb/c mice (20 g, from Charles River, Margate, Kent, UK) were randomly assigned to specific treatment and housed under barrier conditions in filter-topped macron cages (5 mice/cage) at the animal housing facility (Gemeenschapelijk Dierexperimenteel Laboratorium, (GDL)) with drinking water and standard laboratory food pellets ad libitum. At the start, 10 mice received a dose of 2.3 × 10⁷ colony-forming units of freshly cultured *E. coli* bacteria diluted with saline in a 100-μl volume by i.p. injection. CIAP was freshly prepared, and 100 μl containing 1.5 units was administered in the tail vein to five mice directly after administration of bacteria. Body temperature was measured before inoculation of bacteria, and at 1-h intervals during the study by rectal thermometry. Animals whose body temperature dropped below 32°C were euthanized. Animals were monitored during the entire study for e.g., body weight, activity, skin condition, and well being.

**Bacterial Cell Culture.** *E. coli* strain 25922 (American Type Culture Collection, Manassas, VA) was cultured for 18–24 h in brain heart infusion medium at 37°C under vigorous shaking. The culture was washed to remove free LPS and diluted in cold saline to a final concentration of 2.3 × 10⁸ colony-forming units/ml and kept on ice until use to prevent uncontrolled bacterial growth. Tenfold dilutions

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**Fig. 1.** CIAP increases the survival of mice injected with a lethal dose of *E. coli* bacteria (strain 25922; ATCC). At *t* = 0, 2.3 × 10⁷ bacteria were injected i.p. with (open symbols, dashed lines, *n* = 5) or without (closed symbols, solid lines, *n* = 5) simultaneous i.v. administration of 1.5 units CIAP. Asterisks denote significant difference between the group injected with CIAP and the control group (*+, p < 0.05; **+, p < 0.01*).

**Fig. 2.** Plasma elimination curves of CIAP after a single i.v. bolus administration of 400 (*n* = 5) or 4000 (*n* = 6) units. At different time points after CIAP administration, plasma AP activity was determined. Values depicted are the mean ± S.D. after correction for basal AP levels.
were plated on Luria-Bertani-agar plates and incubated for 18 ± 2 h at 37°C to affirm the bacterial concentration administered in vivo.

**Piglets.** Female Dutch Landrace × Yorkshire piglets (n = 40) were obtained from Proefaccommodatie de Tolakker (Utrecht University, Utrecht, The Netherlands) at 8 weeks of weaning. Animals were individually housed under conventional conditions at the animal housing facility of the Utrecht University (GDL) with a 12-h light and 12-h dark sequence. Once a day, they received a commercial pig diet served as sludge, whereas drinking water was provided ad libitum. To facilitate blood sampling, the jugular vein of each piglet was catheterized using two silicon catheters with diameters of 5 and 7 French, respectively, (Sil-C50, Sil-C70; Solomon Scientific/UNO, Zevenaar, The Netherlands) filled with a solution of heparin (10 IU/ml 0.9% NaCl) to prevent blood clotting. During this procedure, animals were sedated with i.m. injection of 10 mg/kg ketamine (Narketan) and 4 mg/kg azaperon (Stressnil), followed by induction of anesthesia with an i.v. injection of 10 to 15 mg/kg thiopental (Nesdoral) and inhalation of a mixture of O2, AIR and 2.5% isoflurane (IsoFlo). The catheter to be used for CIAP administration (5 French) was inserted further into the vein than the one used for taking blood samples (7 French) to prevent mixture. After surgery, piglets were allowed to recover for 1 week during which they received antibiotics [7.5 mg/kg ampicillin (Praxavet) and 1.5 mg/kg flunixine (Fimadrin)].

**Blood Sampling**

Blood sampling through catheter was carried out under aseptic conditions. Catheter content (dead volume) was removed before blood samples were drawn. For the pharmacokinetic experiments, blood samples were collected every 2 min during the first 20 min, up to 1 h every 10 min and every hour up to 6 h after onset of the experiment. Blood samples of the pharmacodynamic experiments were collected every 10 min during the first 1.5 h and every hour up to 6 h after onset of the experiment. After every blood sample, the catheter was filled with heparin to prevent blood clotting.

**Piglet Experiments.** To obtain i.v. bolus pharmacokinetic data, 400 (n = 5) or 4000 (n = 6) units CIAP were suspended in 1 ml of sterile 0.9% saline solution and administered i.v. as a single shot after which blood samples were drawn. To obtain pharmacokinetic data during and after i.v. infusion of CIAP, 2500 (n = 5) or 35,000 (n = 4) units CIAP were suspended in 35 or 40 ml of 0.9% sterile saline solution and administered over 50 or 60 min with an infusion rate of 0.5 ml/min, resulting in a final plasma level of 400 or 4000 units/l, respectively.

To study the safety of repeated CIAP administration, piglets were given CIAP each day for 28 consecutive days (n = 5). For this purpose, 4000 units of CIAP were suspended in 1 ml of sterile 0.9% saline solution and administered as a single i.v. bolus injection. Before each CIAP administration, blood samples were drawn on which AP activity was determined. Twice a week, blood samples were screened for hematological parameters, liver and/or kidney damage, and electrolytes.

Pharmacodynamics of CIAP-LPS interactions were investigated using LPS diluted in sterile 0.9% saline solution to different concentrations and administered as a single i.v. bolus (n = 4) or infusion over 5 to 10 min (n = 15). Animals were given CIAP either as a single i.v. bolus before LPS administration (n = 4) or as an infusion during LPS administration (n = 8). Blood samples were screened for hematological parameters and liver and/or kidney damage. Animals were sacrificed 24 h after LPS administration.

**Clinical Parameters**

In addition to blood sampling, animals were monitored for temperature, heart rate, breathing rate, and well being during the entire course of the experiment.

**Determination of AP Activity**

All blood samples were centrifuged (1500 rpm, 10 min, 15°C) after which the plasma was harvested and AP activity was determined. In brief, 5 μl of plasma was incubated for 30 min at 37°C with 200 μl of assay mix containing incubation buffer (0.025 M glycine/NaOH, pH 9.6), 4-nitrophenylphosphate, and MgCl2 at final concentrations of 1.25 and 2 mM, respectively. The enzyme reaction was stopped by adding 1 ml of 0.1 M NaOH after which the end product p-nitrophenol was quantitatively determined by measuring the extinction at 405 nm.

**Hematological Parameters and Clinical Chemistry**

Hematological and clinical chemistry parameters were data-logged at the clinical hematology facility of the Faculty of Veterinary Medicine (Utrecht University). All blood samples were screened for hematological parameters (number of WBCs by auto-analyzer, WBC differentiation by May-Grunwald staining and light microscopy, and thrombocytes by counting in Bürker chamber by phase contrast microscopy) and liver and/or kidney damage (ureum, creatinine, and γ-glutamyltransferase by auto-analyzer). Blood samples of the 28-day safety study were also screened for electrolytes (sodium, potassium, chloride, calcium, magnesium, and phosphate by auto-analyzer).

**TABLE 1**

<table>
<thead>
<tr>
<th>Dose (units)</th>
<th>t½ 1st (min)</th>
<th>t½ 2nd (min)</th>
<th>AUC 1st (U • min/l)</th>
<th>AUC 2nd (U • min/l)</th>
<th>Vd (liter)</th>
<th>CF (1/min)</th>
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<tr>
<td>400</td>
<td>2.31 ± 0.50*</td>
<td>144 ± 82</td>
<td>2599 ± 2245</td>
<td>1213 ± 499</td>
<td>1.16 ± 0.22</td>
<td>0.27 ± 0.22</td>
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<tr>
<td>4000</td>
<td>3.52 ± 0.46**</td>
<td>201 ± 102</td>
<td>7878 ± 3960*</td>
<td>19,922 ± 15,548*</td>
<td>1.90 ± 0.89</td>
<td>0.34 ± 0.14</td>
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</tbody>
</table>

*p < 0.05 for 4000 versus 400 units.

*Initial phase of elimination curve.

**Second phase of elimination curve.

*Initial clearance.
were seeded per well in 96-well flat-bottom microtiter plates. The porcine kidney cell line PK15 (CCL 33) was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium with glutamax I (Invitrogen, Breda, The Netherlands) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 10% fetal calf serum, and 2% porcine serum. At confluence, cells were split 1:2 with 0.05% trypsin/EDTA (Invitrogen, Breda, The Netherlands). Cells were seeded at 5 ×10^4 cells/ml in 5 ml of PK15 medium. After 16 ± 2 h culture at 37°C, 5% CO2, medium was removed and substituted with 50 μl of PK15 medium supplemented with 2 μg/ml actinomycin-D (Acros, Geel, Belgium). After 2-h culture at 37°C, 5% CO2, medium was added to each well. The plates were incubated at 37°C, 5% CO2 for an additional 16 ± 2 h. Cells were fixed for 10 min with 50 μl of 25% acetic acid, 75% methanol (WVR, Amsterdam, The Netherlands) and stained during 30 min with 50 μl of 0.4% crystal violet (Acros) in methanol. Plates were rinsed with distilled water, dried at 50°C for 4 h, and optical densities were determined spectrophotometrically at 595 nm.

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Days after Initial Injection</th>
<th>Reference Values</th>
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<tr>
<td>Leukocytes (× 10^9/l)</td>
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<td>1.1–22</td>
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<tr>
<td>Lymphocytes (× 10^9/l)</td>
<td>0</td>
<td>0.7–1.1</td>
</tr>
<tr>
<td>Thrombocytes (× 10^9/l)</td>
<td>0</td>
<td>370–32</td>
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<tr>
<td>Ureum (mmol/l)</td>
<td>0</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>Creatinin (μmol/l)</td>
<td>0</td>
<td>141 ± 5</td>
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<tr>
<td>γ-Glutamyltransferase (U/l)</td>
<td>0</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>0</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>0</td>
<td>4.4 ± 0.5</td>
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<tr>
<td>Chloride (mmol/l)</td>
<td>0</td>
<td>104 ± 1</td>
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<tr>
<td>Calcium (mmol/l)</td>
<td>0</td>
<td>2.6 ± 0.1</td>
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<tr>
<td>Magnesium (mmol/l)</td>
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<td>0.84 ± 0.07</td>
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<tr>
<td>Phosphate (mmol/l)</td>
<td>0</td>
<td>2.55 ± 0.30</td>
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### Table 3

Safety parameters after daily administration of 4000 units of CIAP for 28 consecutive days (n = 5). Reference values were obtained from the Animal Health Laboratory (University of Guelph, Ontario, Canada http://ahl.uoguelph.ca/UsersGuide).

**Data Analysis**

The plasma concentration curves of CIAP were fitted using the pharmacokinetic program Multifit (Department of Pharmacokinetics and Drug Delivery, University Center for Pharmacy, University of Groningen, The Netherlands). Data are depicted as mean ± S.D. Statistical analysis was performed using chi square test for mice survival experiments, two-sided unpaired Student’s t test for pharmacodynamic experiments in piglets, and 95% confidence intervals for pharmacodynamic experiments in piglets (*p < 0.05, **p < 0.01, and ***p < 0.001).

### Results

**Effects of CIAP Treatment on Mice Injected with a Lethal Dose of Bacteria.** To investigate the potentially therapeutic effects of CIAP in vivo, mice received a lethal dose of E. coli bacteria i.p. followed by either an i.v. injection of 1.5 unit CIAP (n = 5) or saline (n = 5). Figure 1 shows that four of five animals that were given saline died, or body temperature dropped below 32°C between 10 and 30 h after inoculation of bacteria. In contrast, only one of five animals receiving CIAP after inoculation of bacteria died between 36 and 48 h. Forty-eight hours after bacterial challenge, body temperature of CIAP-treated animals was back to normal (36.2 ± 0.7°C), whereas the animals receiving saline showed a significantly lower body temperature (34.2 ± 0.8; p < 0.05).

**Basal AP Levels in Piglets.** Before the pharmacokinetic experiments in piglets were started, basal plasma AP levels in two nontreated animals were determined (40 ± 7 U/l). Based on these results, the doses to be used in the pharmacokinetic experiments were 400 and 4000 U of CIAP, comparable with 10 or 100 times basal AP levels, respectively.
Pharmacokinetic Bolus Experiments. To study the pharmacokinetic behavior of CIAP, piglets received a single i.v. bolus administration of 400 (n = 5) or 4000 (n = 6) units CIAP. At different time points after CIAP administration, plasma AP activity was determined. As shown in Fig. 2, the plasma elimination curve of CIAP consists of two phases. Kinetic parameters were therefore calculated according to a two-compartment model and listed in Table 1.

Pharmacokinetic Infusion Experiments. Given the fast elimination of CIAP after a single bolus administration, infusion experiments were performed, resulting in final CIAP steady-state plasma levels of 400 (n = 5) or 4000 (n = 4) units/l, respectively within half an hour. At different time points before and after CIAP infusion, plasma AP activity was determined. Figure 3 shows that the plasma elimination curves of CIAP after steady state also consist of two distinct phases. Kinetic parameters are summarized in Table 2.

Multiple Dosing Experiments. Given the short t1/2 of CIAP in plasma, it may be required for clinical application to apply repeated or continuous dosing regimes in LPS overload conditions such as sepsis. Therefore, five piglets received a single i.v. bolus administration of 4000 units CIAP every day for 28 consecutive days. Every day, blood samples were tested on plasma AP activity and safety parameters were assessed twice a week. The results of the safety study are summarized in Table 3. Administration of high doses of CIAP during 28 consecutive days had no statistical significant in-...
fluence on hematological parameters, clinical chemistry, and electrolyte balance.

Effects of CIAP Treatment on LPS-Induced Changes in Clinical Parameters. LPS-induced effects on clinical parameters showed to be dose-dependent and were back to normal 5 to 6 h after LPS administration, whereas LPS + CIAP-treated animals showed a quicker recovery (data not shown).

Effects of CIAP Treatment on LPS-Induced Hematological Changes in Piglets. Because LPS administration is known to cause hematological changes in which high doses result in decreased leukocyte counts and low doses result in leucocytosis, the effect of LPS administration on blood cells was studied. Indeed, 10 μg of LPS/kg reduced leukocyte counts, whereas 50 ng of LPS/kg caused an increase thereof (Table 4). Differential WBC counts showed a right-shift (p < 0.05) at 2 h (Fig. 4A) and a left-shift (p < 0.05) at 5 h (Fig. 4B) after 10 μg of LPS/kg, whereas at both time points left-shifts were observed after administration of 50 ng of LPS/kg (p < 0.05). Although CIAP had no effect on the decrease in leukocyte counts at both time points after a high LPS dose, the left-shift 5 h after a high dose of LPS was completely repressed. The effects of 50 ng of LPS/kg were completely repressed by 4000 units of CIAP (Fig. 4; Table 4). The effects of 4000 units CIAP with 10 μg or 50 ng of LPS/kg on differential blood cell counts are comparable with, respectively, 2 μg or 10 ng of LPS/kg alone (hatched bars). Thrombocyte counts were not clinically relevantly affected by the administration of low LPS doses. High LPS doses, however, resulted in decreased thrombocyte counts (19 ± 1), an effect that was significantly reduced by 2500 units CIAP (41 ± 4; p < 0.05). Together, the above-mentioned results show that administration of CIAP resulted in up to 80% neutralization of the administered LPS.

Dose-Effect Curves of LPS on TNFα Release in Piglets. To investigate the in vivo effects of LPS on the immune system, four piglets were injected intravenously with various doses of LPS (one piglet per dose) and screened for TNFα levels at various time intervals. As shown in Fig. 5, TNFα plasma levels were detected in all piglets injected with LPS with a dose-dependent maximum at approximately 1 h after injection. Two to three hours after LPS administration, TNFα levels were back to normal.

Effects of CIAP Treatment on LPS-Induced TNFα Release in Piglets. Results show a clear difference in TNFα release in piglets treated with LPS alone compared with piglets treated both with LPS and CIAP (Fig. 6). Comparison of TNFα activity over time (AUC) showed that infusion of 2500 units CIAP over 50 min reduces TNFα release after 10 μg of LPS/kg body weight by 75% (Fig. 6A). TNFα release after an infusion of 200 ng of LPS/kg body weight is inhibited by 98% when administered during an infusion of 3000 units CIAP (Fig. 6B). Simultaneous administration of 60 units CIAP and 50 ng of LPS/kg body weight results in an inhibited TNFα release of 44% compared with TNFα release after 50 ng of LPS/kg body weight administration (Fig. 6C).

In Fig. 7, TNFα AUC is plotted against LPS dose. In the low-dose range, a 5-fold increase in LPS dose results in a 250-fold increase in TNFα activity over time, whereas in the highest dose range a 5-fold increase in LPS dose only results in a 4-fold increase in TNFα activity over time. The relative amount of LPS that is dephosphorylated and thereby detoxified by CIAP can be deduced from Fig. 7. This is done by projecting the TNFα response of LPS + CIAP on the LPS
dose-response curve and by reading the corresponding LPS dose. The results show that CIAP can neutralize up to 80% of the administered LPS.

**Discussion**

Poelstra et al. (1997a,b) showed that LPS is a substrate for AP and suggested its role in protection against endotoxin insult typical for Gram negative bacteria. They demonstrated that human placental alkaline phosphatase is able to detoxify LPS by dephosphorylation of the lipid A moiety (Verweij, personal communication). This results in its dephosphorylated counterpart monophosphoryl lipid A, which was shown to be nontoxic and nonpyrogenic by Takayama et al., (1984a,b). If exogenous administered AP in vivo would also result in detoxification of LPS, this may imply that AP candidates for a novel therapeutic agent in the treatment of Gram negative bacterial insult. Experiments performed by Koyama et al. (2002) showed that rat intestinal AP is able to detoxify LPS in vitro and in vivo.

The aim of the present study was to investigate the possible use of CIAP as a therapeutic agent that attenuates LPS-mediated diseases. Toward this goal, mice were injected with a lethal concentration of E. coli bacteria alone or in combination with 1.5 units of CIAP. As a result of LPS administration, body temperature was significantly decreased, which was previously reported by Kozak et al. (1995). The resulting strongly reduced mortality warranted for further study in a LPS piglet model. Furthermore, pharmacokinetic and safety parameters after single dose, multiple dose, and steady-state administration of CIAP were determined.

Independent of the administered dose, a biphasic clearance with a fast initial and a slower second elimination of CIAP from the circulation was found. This is in agreement with results obtained by Scholtens et al. (1982), who performed their experiments with canine intestinal AP in rats, and Hoffmann et al. (1977), who studied the kinetic behavior of feline and canine intestinal AP in cats and dogs, respectively.

Because of the short initial half-life of CIAP, its elimination from plasma is mainly determined by the initial phase of the curve, which approximately encompasses the first 10 min after CIAP administration. Although 10 min after CIAP administration 80 to 90% of the administered CIAP is cleared from the plasma, in the second phase, CIAP activity accounts for up to 80% of total enzyme activity over time (Tables 1 and 2; AUC second phase relative to total AUC).

Being a nonsialylated glycoprotein, CIAP is likely to be cleared by the asialoglycoprotein receptor (ASGPR) on liver cells, first discovered by Pricer and Ashwell (Pricer, Jr. and Ashwell, 1971; Pricer, Jr. et al., 1974). Indeed, Scholtens et al. (1982) and W. Verweij (personal communication) showed that the plasma residence time of canine and calf intestinal AP, respectively, could be extended by blocking the ASGPR. However, such ASGPR clearance does not account for the second phase with a plasma half-life time of approximately 2 h. This biphasic kinetic profile was explained by either rate-limiting receptor recycling or bidirectional transport of AP between the liver and the plasma compartment, resulting in reentry of CIAP in the plasma compartment (Scholtens et al., 1982; Kuhlenschmidt et al., 1991). The first explanation, however, should show dose-dependent plasma half-lives times, which we and others (Hoffmann and Dorner, 1977; Hoffmann et al., 1977; Scholtens et al., 1982) did not observe.

Different from most other asialoglycoproteins, however, CIAP possesses a glycosyolphosphatidylinositol anchor. It has been reported by Low and Zilversmit (1980) and Medof et al. (1996) that glycosyolphosphatidylinositol-anchored proteins bind to cell membranes. Due to the huge surface of the blood vessel endothelium, we hypothesize that administered CIAP binds to these endothelial cells in the first phase of clearance, resulting in a fast initial disappearance of CIAP from the plasma. In the second phase, release of AP from the endothelial wall into the bloodstream would result in much slower plasma elimination.

Due to the pharmacokinetic profile of CIAP, a multiple dosing regime may apply to obtain suitable plasma AP levels in a clinical setting. It was established that repeated dosing did not affect the health or the hematological and clinical parameters throughout the study period of 28 days (Table 3). In addition, no accumulation of CIAP in the plasma was observed after the 28-day treatment (data not shown).

Having established the pharmacokinetic and safety profile of CIAP, the LPS-detoxifying capacity of CIAP was studied in a piglet model. In this model, LPS was administered intravenously with or without CIAP. The proposed action of AP is dephosphorylation and thereby detoxification of LPS before LPS can exert its toxic effects through TLR signaling. To
prevent the immediate effects of LPS, CIAP was administered before LPS.

Administration of LPS to piglets induces hematological changes (Norimatsu et al., 1995; Andonova et al., 2001), which are mostly effects on WBC differentiation and thrombocytes. Our results show that the dose-dependent effects of LPS on differential WBC counts and thrombocytes are inhibited by CIAP. LPS administration also resulted in a dose-dependent TNFα release in piglets, results that are supported by Norimatsu et al. (1995) and Webel et al. (1997). CIAP administered before LPS inhibited TNFα release up to 98%.

In fact, two models were used, one for sepsis and one for inflammation, corresponding to high and low LPS dose administration, respectively. In the sepsis model, 10 μg LPS/kg induced signs of severe sepsis, as specified in the APACHE score table (Knaus et al., 1985). These included tachycardia, fever, low WBC counts, and low thrombocyte counts. In contrast, 50 ng of LPS/kg did not show signs of severe sepsis but signs of inflammation, such as fever, leukocytosis, and cytokine induction.

In both the sepsis and the inflammation model, CIAP attenuates LPS toxicity. A striking observation in both models and for most parameters was that the effectiveness of LPS + CIAP was similar to that of 20% of that LPS dose, meaning that CIAP detoxified 80% of the administered LPS. Another explanation for the overall 80% less toxicity may be that dephosphorylated LPS is 80% less toxic than LPS itself. For lipid A, it has already been shown that after its dephosphorylation it completely lost toxicity, but for dephosphorylated lipid A, it has already been shown that after its dephosphorylation it completely lost toxicity, but for dephosphorylated LPS, the remaining toxicity has never been investigated.

Together with the observed effects of CIAP administration on LPS-mediated changes in differential WBC counts, thrombocyte counts, and TNFα release, the present study shows that CIAP neutralizes and thereby detoxifies LPS for up to 80%. Although other anti-sepsis therapies aim at anti-inflammation or maintain critical organ function, the function of CIAP is to detoxify the inflammatory agent, LPS, itself.

Furthermore, the physiological function of alkaline phosphatase may be that of dephosphorylating and thereby detoxifying LPS, so the addition of CIAP in LPS overload conditions such as sepsis may add physiological aid in combating excess LPS before harmful effects may occur. In all experiments CIAP was administered before LPS exposure. This was done to prevent the immediate LPS toxicity through interaction with the respective LPS receptor (Treon et al., 1994). We stress that alkaline phosphatase does not interact with the ongoing LPS toxic cascade but rather prevents follow-up toxicity of newly released LPS. Therefore, it is proposed that CIAP represents a novel therapeutic drug in the treatment of Gram negative sepsis and other LPS-mediated diseases. Based on the results of the present study, human clinical trials have been performed.

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References


