American Journal of Pathology, Vol. 151, No. 4, October 1997
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Dephosphorylation of Endotoxin by Alkaline Phosphatase *in Vivo*

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Natural substrates for alkaline phosphatase (AP) are at present not identified despite extensive investigations. Difficulties in imagining a possible physiological function involve its extremely high pH optimum for the usual exogenous substrates and its localization as an ecto-enzyme. As endotoxin is a substance that contains phosphate groups and is usually present in the extracellular space, we studied whether AP is able to dephosphorylate this bacterial product at physiological pH levels. We tested this in intestinal cryostat sections using histochemical methods with endotoxin from *Escherichia coli* and *Salmonella minnesota* R595 as substrate. Results show that dephosphorylation of both preparations occurs at pH 7.5 by AP activity. As phosphate residues in the lipid A moiety determine the toxicity of the molecule, we examined the effect of the AP inhibitor levamisole *in vivo* using a septicemia model in the rat. The results show that inhibition of endogenous AP by levamisole significantly reduces survival of rats intraperitoneally injected with *E. coli* bacteria, whereas this drug does not influence survival of rats receiving a sublethal dose of the gram-positive bacteria *Staphylococcus aureus*. In view of the endotoxin-dephosphorylating properties of AP demonstrated *in vitro*, we propose a crucial role for this enzyme in host defense. The effects of levamisole during gram-negative bacterial infections and the localization of AP as an ecto-enzyme in most organs as well as the induction of enzyme activity during inflammatory reactions and cholestasis is in accordance with such a protective role. *(Am J Pathol 1997, 151:1163–1169)*

Alkaline phosphatase (AP) is a membrane-bound enzyme that can dephosphorylate various phosphorylated substrates.[1,2] Although this phosphatase has been studied for some decades and is found in many tissues,[3] its natural substrate is still unidentified.[1,4,5] The enzyme exhibits an unphysiological pH optimum of 10.5 for the conversion of several substrates,[6] and its location on the plasma membrane as an ecto-enzyme[7] makes a function in cell metabolism unlikely.[1]

Endotoxin is a product of gram-negative bacteria and is abundantly present in the external environment and in the intestinal lumen tract.[8] This lipopolysaccharide (LPS) elicits fulminant inflammatory reactions that may be lethal, particularly when the compound enters the general circulation.[9] The toxic moiety of LPS (lipid A) contains two phosphate groups[10–12] that are considered to be essential for its biological actions.[11,13] Monophosphoryl lipid A is only a weak activator of macrophages and is much less toxic as compared with diphosphoryl lipid A.[13] In fact, we found that exogenous AP is able to attenuate the inflammatory response upon LPS in rats[14] and mice.[15]

In the present study we tested whether intestinal AP is able to dephosphorylate wild-type LPS from *Escherichia coli* as well as LPS from *Salmonella minnesota* containing only phosphate groups in the lipid A moiety. LPS dephosphorylation by AP was studied at physiological pH levels. Moreover, we examined whether inhibition of endogenous AP by levamisole would alter the sensitivity for LPS in rats. The results clearly support the hypothesis that endotoxin is a natural substrate for AP. This idea is further supported by data from studies with levamisole *in vivo* and by observations regarding the localization and induction of enzyme activity during pathological processes.

Materials and Methods

Animals and Tissue Sampling

Wistar rats (male, 200 to 220 g) were obtained from Harlan CPB (Zeist, The Netherlands). Rats were housed under specific-pathogen-free (SPF) conditions. Tissue samples from these animals were randomly selected, and two samples from each organ were placed adjacent to each other and snap-frozen in isopentane (−80°C). Consequently, one cryostat section comprises two tissue samples.

Accepted for publication July 3, 1997.

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LPS Preparations

LPS from *E. coli* serotype 0.55:B5 (BioWhittaker, Walkersville MD) and from *S. minnesota* R595 (List Biological Laboratories, Campbell CA) was used in the present study. To exclude the presence of phosphate-containing contaminants, total phosphate content of each batch was determined according to the method of Böttcher[16] and related to the LPS content.

Histochemical Detection of AP Activity

AP activity was demonstrated at pH 9.0 according to standard procedures (method of Gomorri,[17] in cryostat sections of four rats. Sections of 4 μm were incubated with 20 mmol/L β-glycerophosphate as substrate (Merck, Darmstadt, Germany) for 1 hour at 37°C.

Histochemical Detection of LPS Dephosphorylation

Phosphate release from LPS at a neutral pH level was demonstrated in cryostat sections of four rats according to the method of Wachstein and Meisel.[18] Briefly, sections were fixed in 4% formalin-macrodex and subsequently incubated at 37°C with 0.5 mg/ml LPS from *E. coli* or from *S. minnesota* R595 in 0.2 mmol/L Tris/HCl buffer (pH 7.5). The incubation medium contained also 3.6 mmol/L $Pb(NO_3)_2$ and 10 mmol/L $MgSO_4$. After 1 hour, lead phosphate precipitates were visualized by conversion to lead sulfide. Some of the stainings were carried out in the presence of an inhibitor of intestinal AP, L-phenylalanine (5 mmol/L). Control incubations were performed without substrate.

To examine LPS dephosphorylation at an alkaline pH level, the method of Gomorri was applied with LPS as substrate. Sections were incubated with 0.5 mg/ml LPS from *S. minnesota* R595 in veronal/$CaCl_2$ buffer, pH 9.0, supplemented with $MgSO_4$. Calcium phosphate precipitates were converted to, respectively, cobalt phosphate and cobalt sulfide according to standard procedures.[17]

LPS dephosphorylation at acidic pH (ie, 5.0) was studied using standard methods for detection of acid phosphatase (method of Gomorri[19]) with LPS from *S. minnesota* R595 (0.5 mg/ml) as substrate instead of the conventional substrate β-glycerophosphate.

Semiquantitative evaluation of each histochemical was performed upon four sections obtained from four rats.

Biochemical Detection of LPS Dephosphorylation

LPS dephosphorylation by AP was assayed by measurement of inorganic phosphate (μmol/L P_i) release. Calf-intestinal AP (1773 U/mg protein; Calbiochem-Novabiochem International, San Diego CA) was incubated for 1 hour at 37°C with LPS from *E. coli* (Biowhittaker) or LPS from *S. minnesota* R595 (List) in 0.05 mmol/L 2-amino-2-methyl-1,3-propanediol buffer with 2 mmol/L $MgCl_2$ at pH 7.8. As equimolar concentrations cannot be achieved, LPS concentrations were standardized to 0.2 mg/ml for both LPS types. Subsequently, P_i concentrations were measured according to the method of Chandrarajan[20] with the modification that no sodium dodecyl sulfate was added to the medium. This procedure allows detection of free P_i without disturbances by LPS-bound phosphate groups. Each assay was performed in triplicate, and results are expressed as arithmetic means of five assays (±SD). Differences were considered significant at $P < 0.05$ (Wilcoxon test).

Effect of Inhibition of AP Activity in Vivo

At t = 0 hour, rats received either 1.0×10^{10} colony-forming units (CFU) of *E. coli* bacteria (ATCC 25922), or 1.0×10^{10} CFU of *Staphylococcus aureus* bacteria (ATCC 29213) or 1.0 ml of vehicle intraperitoneally (n = 8 per group). An additional group of 10 rats received *E. coli* bacteria and the AP inhibitor levamisole[21] (Sigma Chemical Co., St. Louis, MO), whereas another group of rats (n = 8) received *S. aureus* bacteria plus levamisole. Levamisole was administered subcutaneously at t = −2 hours in a dose of 50 mg/kg body weight. Pilot experiments showed that this dose caused a reduction of approximately 50% in plasma AP activity 6 hours after administration.

Rectal body temperature was measured at t = 5 hours, and body weight was monitored during the whole observation period (5 days).

AP Activity in Suspensions of Neutrophils

Neutrophils were isolated according to standard centrifugation methods[22] on a Lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway) from blood obtained from healthy male volunteers. Volumes were adjusted to a cell concentration of 1.0×10^7 cells per ml. Purity of the suspensions was checked by light microscopy.

Cells were incubated for 30 minutes at 37°C with 20 pg/ml LPS from *E. coli* or with vehicle (0.9% NaCl). Subsequently, AP activity of the cellular suspension was measured according to standard methods at pH 9.8 with 4-nitrophenylphosphate as substrate.[6] When indicated, levamisole (Sigma Chemical Co.) was added to the incubation medium in a final concentration of 0.2 mmol/L.

AP Activity During Cholestasis

Cholestasis was induced by bile duct ligation[23] under halothane, O_2/N_2O anesthesia. Control rats received a sham operation. Cholestasis was confirmed by weekly measurements of serum bilirubin levels (>100 μmol/L). Three weeks after surgery, all animals (four rats per group) were sacrificed and liver samples were snap frozen at −80°C. Cryostat sections of these livers were examined for AP activity and LPS dephosphorylating activity as described above.

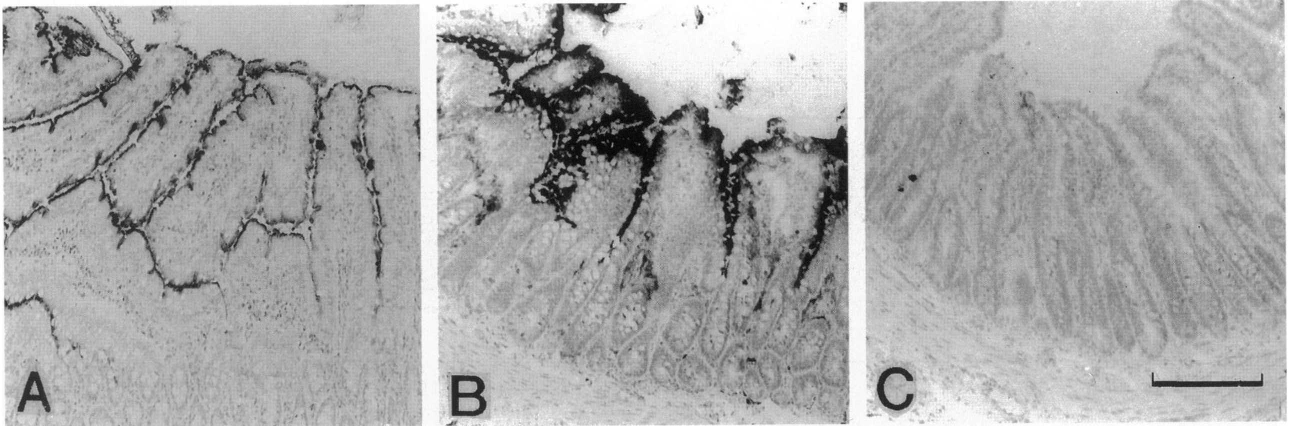

Figure 1. Reaction product (dark staining), reflecting phosphatase activity, in intestinal cryostat sections with either LPS from E. coli *as substrate* (**A**) or β-glycerophosphate (**B** and **C**). LPS concentration was 50 μmol/L (pH 7.5) whereas β-glycerophosphate was applied in concentrations of 20 mmol/L (**B**) and 50 μmol/L (**C**), both at pH 9.0. Note the identical staining patterns in **A** and **B** and the strong staining intensity with LPS as substrate as compared with β-glycerophosphate at equal substrate concentrations (**A** versus **C**). Magnification, ×64; scale bar, 200 μm.

Results

First, total P_i content of the LPS batches was measured. LPS from *E. coli* contained 4.2 μmol of P_i per μmol of LPS (M_r of wild-type LPS is approximately 10,000,[11,12] whereas LPS from *S. minnesota* R595 contained 2.1 μmol of P_i per μmol of LPS; the molecular weight of lipid A with three 2-keto-3-deoxyoctonate (KDO) sugars is 2305.[11,12] These molar phosphate:LPS ratios excluded the presence of significant amounts of phosphorylated contaminants and/or phosphates, and consequently these batches were applied in the studies described here.

Staining of rat intestinal cryostat sections for phosphatase activity at pH 7.5, with LPS from *E. coli* as substrate, invariably yielded a marked black staining (reflecting P_i release) along the epithelial layer of intestinal crypts in all four rats examined (Figure 1A). An identical staining pattern was observed when LPS from *S. minnesota* R595 was applied as substrate (see also Table 1). This pattern corresponded with the localization of AP activity, as demonstrated using conventional methods at pH 9.0 with the substrate β-glycerophosphate (Figure 1B), although staining intensity was considerably higher in these latter sections. However, when β-glycerophosphate concentration was reduced from 20 mmol/L (standard procedures[17]) to 50 μmol/L (equimolar to wild-type LPS concentrations), no reaction product was found in any of the sections examined (Figure 1C).

Table 1. Semiquantitative Evaluation of Phosphatase Activity in Intestinal Cryostat Sections at Various pH Levels with Several Substrates

	LPS from:			
	E. coli	*S. minnesota*	β-gP	None
pH 5.0	−	−	+++	−
pH 7.5	++	+	+++	−
pH 9.0	−	−	+++	−
pH 7.5 with L-phenylalanine	±	ND	+	−

β-gP, β-glycerophosphate; −, none; +, moderate; ++, medium; +++, strong staining; ND, not done.

L-Phenylalanine, an inhibitor of intestinal AP,[21] attenuated β-glycerophosphate dephosphorylation as well as dephosphorylation of LPS in all cryostat sections (Table 1). No LPS dephosphorylation was found at pH 9.0 and 5.0 using the method of Gomorri (Table 1).

Biochemical assessment of phosphate release from LPS by highly purified preparations of intestinal AP confirmed the histochemical results (Figure 2). Whereas 48.7 ± 26.3 μmol/L P_i was detected in samples with LPS from *E. coli* incubated without AP, 108.0 ± 11.7 μmol/L P_i was found in samples containing intestinal AP and LPS from *E. coli* ($P < 0.005$). Intestinal AP itself was free of phosphate (0.00 ± 13.7 μmol/L P_i). LPS from *S. minnesota* was also dephosphorylated by AP; non-enzymatic P_i release was 60.4 ± 8.6 μmol/L P_i, and AP-induced P_i release was 132.4 ± 23.9 μmol/L P_i ($P < 0.005$). Given the molarities of LPS-containing samples, which is approximately 20 μmol/L for *E. coli* LPS and 87 μmol/L for *S. minnesota* LPS, it can be estimated that an average of 3.0 phosphate groups were enzymatically released from

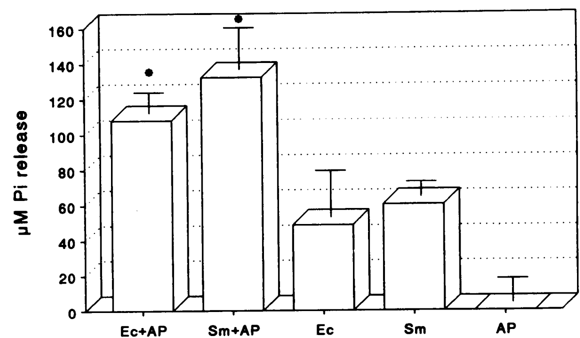

Figure 2. Inorganic phosphate release (μmol/L Pi) by calf-intestinal alkaline phosphatase (200 U/ml) from LPS derived from *E. coli* (Ec; calculated LPS concentration, 20 μmol/L) and from *S. minnesota* R595 (Sm; LPS concentration, 87 μmol/L). As equimolar concentrations cannot be achieved for technical reasons, LPS concentrations were standardized to 0.2 mg/ml for both LPS types. Results show arithmetic means (±SD) of three assays. Parallel incubations were carried out with either LPS from *E. coli* or *S. minnesota* alone or with AP in vehicle. The data show that both LPS preparations are dephosphorylated by purified intestinal AP preparations. *$P < 0.005$ as compared with incubations without AP, Wilcoxon.

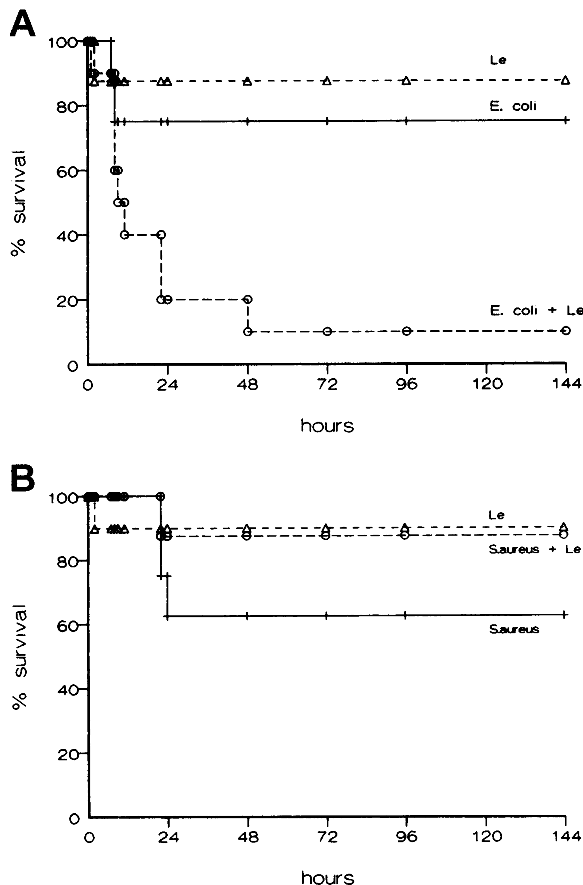

Figure 3. Survival time of rats after administration of bacteria or levamisole or both. **A** Survival after inoculation of *E. coli* bacteria at t = 0 (Ec). **B:** Survival time after administration of *S. aureus*. Some of the animals were treated with levamisole (Le) at t = −2 hours. It is shown that levamisole reduces survival after administration of *E. coli* ($P < 0.005$, χ^2 test) but not after inoculation of *S. aureus*.

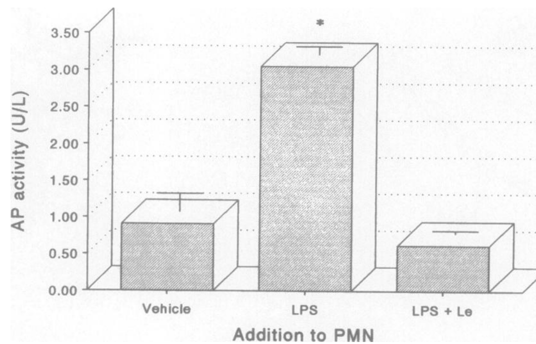

Figure 4. Effect of LPS upon alkaline phosphatase activity of human peripheral blood neutrophils. Results show arithmetic means of four assays (±SD). Either LPS from *E. coli* (20 pg/ml) or LPS with levamisole (le) or vehicle alone was added to the cell suspensions and AP activity was subsequently assayed at pH 9.8 with 4-nitrophenylphosphate as a substrate. *$P < 0.025$ as compared with vehicle, Wilcoxon test.

E. coli LPS and 0.8 phosphate groups from *S. minnesota* LPS.

When endogenous AP activity was inhibited by administration of levamisole (Figure 3), animals showed no apparent signs of intoxication or abnormal behavior; the particular rats were active and body weight remained stable in the observation period (n = 8). Rectal body temperature 5 hours after administration was 36.9 ± 0.6°C and not significantly different (Wilcoxon test) from untreated control rats (n = 8), which exhibited a temperature of 37.4 ± 0.3°C. Rats receiving *E. coli* bacteria (n = 8) had an average body temperature of 36.9 ± 1.2°C (no significant difference as compared with untreated rats, Wilcoxon test) and 25% of the animals died within the observation period. However, when levamisole and *E. coli* bacteria were administered concurrently (n = 10), 90% of the animals died within 24 hours after the injection ($P < 0.005$, χ^2 test). Mean rectal body temperature 5 hours after inoculation of bacteria was 34.9 ± 1.5°C ($P < 0.005$ as compared with untreated rats, Wilcoxon).

As can be seen in Figure 3B, this effect of levamisole was observed only in rats treated with gram-negative bacteria. Survival in the group of rats inoculated with the gram-positive *S. aureus* was 62.5% (n = 8) whereas survival in the group receiving *S. aureus* plus levamisole (n = 8) was 87.5% (no significant difference, χ^2 test).

The expression of AP activity in human neutrophils was markedly increased in the presence of LPS; AP activity in suspensions of isolated neutrophils was 347 ± 83% enhanced after addition of 20 pg/ml LPS to the incubation medium as compared with cell suspensions treated with vehicle (Figure 4).

In vivo, LPS in the bloodstream is predominantly taken up by the liver.[23,24] No LPS dephosphorylation could be detected in cryostat sections of livers from control rats (results not shown). However, after experimental induction of cholestasis, resulting in an enhanced intrahepatic AP expression,[25] LPS dephosphorylating activity was found in the livers of all four rats examined (Figure 5A). Enzyme activity was found at pH 7.5 in portal areas that showed abundant expansion of the extracellular matrix (approximately 50% of the portal areas present in one section). Again, expression of the endotoxin dephosphorylating activity corresponded well with the localization of AP activity as demonstrated using standard methods (Figure 5B), although staining intensity was higher when the method of Gomorri was applied with β-glycerophosphate as substrate.

Discussion

The endotoxin molecule is located in the cell wall of gram-negative bacteria, and this compound is extremely toxic to most mammals. It causes an array of serious clinical problems in man.[9,26] Although the polysaccharide chain of this molecule varies considerably among different bacterial strains, the lipid A moiety is relatively constant and usually contains two phosphate groups.[10,11,27] The molecular structure of this particular region is considered to be essential for the toxicity of endotoxin, and the phosphate groups are important determinations in this respect.[10,13,27]

To test whether AP is able to dephosphorylate endotoxin at physiological pH levels, phosphatase activity was studied at a physiological pH level with LPS as substrate. We tested endotoxin from both *E. coli* and *S. minnesota* to

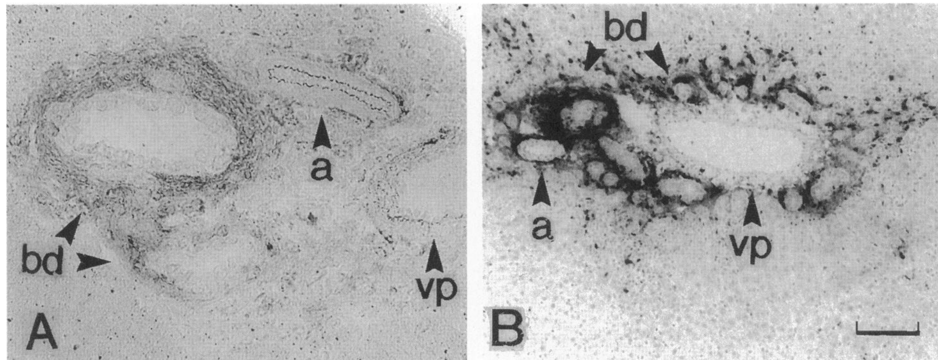

Figure 5. A: Reaction product (dark staining), reflecting LPS dephosphorylation, in a rat liver, 3 weeks after induction of cholestasis by bile duct ligation. **B:** Alkaline phosphatase activity in a cholestatic liver as demonstrated using conventional methods. LPS dephosphorylation is demonstrated according to the method of Wachstein and Meisel with LPS from *E. coli* as substrate. Reaction product can be seen along bile ducts (bd) and hepatic arteries (a), which corresponds with the localization of AP activity (**B**). Vp, vena porta. Magnification, ×40; scale bar, 200 μm.

exclude strain specificity and to exclude that AP dephosphorylates the polysaccharide chain rather than the lipid A moiety; the polysaccharide tail of *S. minnesota* R595 is limited to three 1-keto-3-deoxy-octonate (KDO) sugars.[11,12] Analysis of total phosphate content of LPS preparations confirmed this; the molar phosphate to LPS ratio was 2.1:1 for *S. minnesota* R595, whereas LPS from *E. coli* had an average of 4.2 phosphate molecules per LPS molecule, in line with the presence of two phosphate groups in the lipid A moiety and at least two groups in the polysaccharide chain.[10]

LPS from both *E. coli* and *S. minnesota* was dephosphorylated at a physiological pH level by phosphatase activity in intestinal cryostat sections (Figure 1A and Table 1). The measured enzyme activity was shown to be due to AP because the localization of the reaction product corresponded exactly with AP activity as demonstrated using conventional methods (Figure 1, A *versus* B[28]). Furthermore, enzyme activity was attenuated by the intestinal AP inhibitor L-phenylalanine (Table 1), and highly purified commercially available preparations of intestinal AP enzymes were also able to dephosphorylate LPS *in vitro* (Figure 2).

From biochemical experiments (depicted in Figure 2), it can be estimated that an average of 3.0 phosphate groups are enzymatically released from *E. coli* LPS and 0.8 phosphate groups from *S. minnesota* LPS. LPS from *E. coli* contains approximately 4.2 mol of phosphate per mol of LPS (see above). AP is probably not able to dephosphorylate LPS of *E. coli* completely because wild-type LPS may contain some diesters of phosphate[10] whereas AP hydrolyzes only monophosphate esters.[5] The dephosphorylation of LPS from *S. minnesota* R595 (Re chemotype), which contains only two phosphate groups in the lipid A moiety,[11,12] clearly demonstrates that AP is able to dephosphorylate lipid A. This is particularly important because removal of one phosphate group from the lipid A moiety attenuates the biological effects of the whole LPS molecule.[13,27]

Substrate affinity of AP for LPS was not studied here (kinetic studies are in progress), but it should be noted that LPS concentrations in histochemical studies were relatively low (50 μmol/L) as compared with substrate concentrations in conventional enzyme histochemistry (20 mmol/L[15,28]). This probably explains the difference in staining intensity between the two procedures. When β-glycerophosphate concentrations were reduced to 50 μmol/L, virtually no phosphatase activity was found (Figure 1C).

To test whether inhibition of endogenous AP would lead to an increased sensitivity for LPS *in vivo*, we applied a model of intraperitoneal inoculation of bacteria. This sepsis model leads to an endotoxemia within 6 hours.[29] Nearly all animals pretreated with levamisole died after administration of *E. coli* bacteria, whereas survival was much higher in rats injected with bacteria receiving no levamisole.

Because levamisole is known to have immunomodulatory properties,[30] which in itself might influence death rate after the challenge with bacteria, control groups were infected with gram-positive bacteria. Whereas LPS plays a crucial role in sepsis induced by gram-negative bacteria, sepsis caused by gram-positive bacteria is mediated by other bacterial products such as lipoteichoic acid, muramic acid, and protein A.[31–34] Despite this difference in the initial provocative stimulus, both types of microorganisms stimulate the production of tumor necrosis factor-α, interleukins (IL-1, -4 and -6) and interferon-γ in macrophages, lymphocytes, and other cells.[31–36] *S. aureus* products even bind to CD14, the LPS receptor.[37] As a consequence, identification of the invading microorganism in septic patients based upon cytokine profiles[38,39] or inflammatory responses[38] is very difficult. Accordingly, the same anti-cytokine treatments have been proposed.[40–42] Although the severity of septic shock may be less intense in gram-positive bacteremia,[43,44] it may be expected that the immunomodulatory effect of levamisole also occurs in animals injected with gram-positive bacteria, and consequently this should reduce survival rate as in *E. coli*-treated rats, particularly when bacteria concentrations are high. As levamisole did not reduce survival in *S. aureus*-inoculated rats, we propose that AP inhibition rather than an immunomodulatory effect caused the reduced survival in *E. coli*-treated animals.

An increased serum AP activity is found during inflammatory reactions,[5] cholestasis,[4,25] and pregnancy and in periods of increased bone formation.[1]

As can be seen in Figure 4, LPS directly induced the expression of AP activity in inflammatory cells, as assessed *in vitro* using a suspension of neutrophils. This observation is in line with the proposed role for this enzyme in first-line host defense. This notion is further supported by recent publications showing that AP is localized in specific granules in neutrophils[45,46] and that endotoxin accumulates in these particular granules.[47]

Increased AP activity is also a hallmark of cholestasis in rats and man.[4,26] Elevated AP levels are found both in serum and in liver, where abundant AP activity is induced in the portal area along proliferating bile ducts[48] (see also Figure 5A). Histochemical experiments with livers of cholestatic rats in the present study clearly showed that this AP activity is capable of dephosphorylating LPS from *E. coli* (Figure 5B). The lack of LPS dephosphorylating activity in normal rat livers may be due to the low AP activity in these livers in combination with the relatively low substrate concentration.

Several reports indicate that cholestasis is associated with elevated levels of endotoxin due to an impaired handling of this substance by the liver.[8] It has been found that phagocytic activity of Kupffer cells, the major cell type involved in endotoxin detoxification,[49] is suppressed during cholestasis,[23,49] whereas the normal enterohepatic route for endotoxin removal via the biliary system is also blocked during this disease. In view of the action of AP demonstrated here, it is conceivable that the enhanced AP activity observed during this disease reflects a physiological response of the liver upon bile duct obstruction. Additional experiments are in progress to elucidate this issue.

Endotoxin may not be the only endogenous substrate of AP. Other authors suggested that phosphorylcholine may be a substrate for intestinal AP.[50] In addition, in bone tissue, with a high AP content, anti-endotoxin activity does not seem relevant. There is evidence that AP enzymes directly play a role in the mineralization process in the bone matrix,[51] although its role is not quite clear due to the high pH levels necessary for this enzyme activity.

In summary, the results of the present study demonstrate that LPS can be dephosphorylated by AP at physiological pH levels. In particular, the dephosphorylation of the lipid A moiety, indicated by the phosphate release from Re-LPS from *S. minnesota*, is pathophysiologically important. We hypothesize that for a proper phosphatase activity negative charges are required at the surface of the enzyme,[14,15] dephosphorylation of endotoxin can take place at physiological pH because the LPS exposes multiple negatively charged groups to the active site of the enzyme. In case of the usual exogenous substrates such as β-glycerophosphate and 4-nitrophenylphosphate that lack multiple anionic groups, a high pH level is necessary for an optimal enzymatic activity. We propose that this is to produce negatively charged (dissociated carbonyl) groups in the vicinity of the active site of the enzyme.[15] Studies elaborating this issue are currently in progress.

The endotoxin dephosphorylating activity of AP may reflect an essential physiological function of the enzyme. Data from *in vivo* studies with levamisole and the results of previous studies showing a protective effect of exogenous AP during local inflammatory reactions in rats[14] and mice[15] support this idea. Also, the induction of AP activity *in vivo* during inflammation and cholestasis as well as its constitutive expression at strategic positions in the intestine and neutrophils and along blood vessel walls[17,28] is in accordance with a role for AP in host defense.

Acknowledgments

We thank Mr. Kor Kooi for his supply of bacteria and Mr. Siep Noorman for performing the microphotography. The assistance of Mrs. A.M. van Loenen-Weemaes and Mr. Peter Heeringa is also greatly appreciated.

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