Safety and efficacy of bovine calf intestinal alkaline phosphatase

a novel therapeutic drug for LPS-mediated diseases

Géneviève Chantal Beumer

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Safety and efficacy of bovine calf intestinal alkaline phosphatase

a novel therapeutic drug for LPS-mediated diseases

Veiligheid en werking van bovine kalf intestinaal alkalisch fosfatase

een mogelijk nieuw geneesmiddel tegen LPS-gemedieerde ziekten

(met een samenvatting in het Nederlands)

Proefschrift

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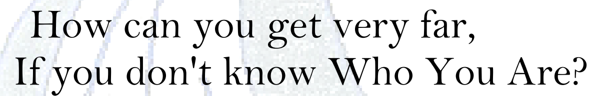

How can you get very far,
If you don't know Who You Are?

How can you do what you ought,
If you don't know What You've Got?

And if you don't know Which to Do
Of all the things in front of you,

Then what you'll have when you are through
Is just a mess without a clue
Of all the best that can come true
If you know What and Which and Who.

(Benjamin Hoff - *the Tao of Pooh*)

Lieve mama, voor altijd …

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1

General introduction

Sepsis and septic shock

When an invading microorganism challenges the human body, a series of defence mechanisms is activated in order to define the invader (e.g. bacteria, viruses or fungi), control the damage and repair the injured tissues and organs. This reaction, which involves certain cells of the immune system, in most cases allows survival of the patient with a minimum of residual damage. In some cases, however, an imbalance between the degree of injury and the host reaction occurs, also called SIRS (systemic inflammatory response syndrome). SIRS often develops to severe sepsis, a state at which bacterial growth is often found and organ dysfunction occurs. Septic shock is defined as a state of hypotension that occurs despite adequate fluid infusion. It is associated with perfusion abnormalities, hypotension, altered mental conditions and multiple organ failure (MOF). The classical criteria for SIRS and sepsis, proposed originally in 1992 and subsequently validated in 1995, are the following [2]:

- ***SIRS (2 or more):***
  - Temperature > 38˚C or < 36 ˚C
  - Heart rate > 90 bpm
  - Respiratory rate > 20 bpm
  - WBC count > 12.000 / mm^3, < 4.000 / mm^3 or > 10% immature cells
- ***Sepsis:*** SIRS + documented infection
- ***Severe sepsis:*** Sepsis + organ dysfunction
- ***Septic shock:*** Sepsis induced hypotension not responding to iv fluid infusion + peripheral hypoperfusion

Sepsis and the accompanying SIRS represent a broad spectrum of clinical symptoms and are characterized by a first phase of hyperresponsiveness (systemic inflammatory response), sometimes resulting in early death, and a much longer phase of hyporesponsiveness (compensatory anti-inflammatory response syndrome; CARS) and immunosuppression. The presence or absence of bacteria in the blood as well as the difference between Gram-positive and Gram-negative organisms seems to have little or no influence on the course or prognosis of the disease.

Microbial sepsis, and Gram-negative sepsis in particular, is still a major clinical problem worldwide and appears to be increasingly common, especially in intensive care units (ICUs) [3]. The incidence of sepsis continues to rise and with the increase in chronic diseases, the use of immunosuppressive therapy, invasive monitoring devices, resistant microorganisms and elderly people, a further increase is expected. At the same time, there is still no therapeutic approach for the immunological imbalance responsible for this disease.

SEPSIS FACTS

- Over 18 million cases of severe sepsis worldwide each year [1]
- Up to 135,000 European and 215,000 American deaths each year [1,4]
- Kills approximately 1400 people worldwide every day [5]
- Severe sepsis is the leading cause of death in the non-coronary ICU [5]
- Each year sepsis costs €7.6 billion in Europe and €17.4 billion in the US [1,5]

Usually, microorganisms co-exist in harmony with the human body and in general serve a beneficial function. Infections result when this normal flora or a pathogenic microorganism causes tissue invasion or an inflammatory response. The commonest sites of infection are the mucosal surfaces of the lungs, abdominal cavity, the urinary tract and primary infections of the blood stream [6]. These barriers are intact in healthy subjects but may be compromised by illness-related factors in hospitalized patients. Here, enhanced colonization can lead to sepsis by direct invasion and/or translocation of bacteria or their products.

A microbiological diagnosis is made in about half the cases. Gram-negative bacteria used to be the major cause of sepsis but since the mid-1980s Gram-positive bacteria have become the predominant cause, accounting for 56% of cases [7]. The incidence of fungal sepsis has increased threefold since 1979, of which most cases are caused by Candida species [8,9]. But while gram-negative bacteria are not the major cause of sepsis, they are the most deadly. Nearly 60% of gram-negative bacteremiae lead to septic shock while only 5% to 10% of patients with gram-positive or fungal bloodstream infections develop sepsis [8]. *E. coli, K. pneumonia, P. aeruginosa, N. meningitides, Proteus* species and *Serratia* species are the gram-negative bacteria of greatest concern.

Gram-negative bacteria, as indicated above, occupy a unique position among sepsis-causing microorganisms. In contrast to Gram-positive bacteria, they contain a functionally important cell membrane exterior of their rigid peptidoglycan cell wall of which the inner leaflet consists of traditional phospholipids and the outer leaflet is composed of lipopolysaccharide (LPS), also known as endotoxin. The presence of LPS and its ability to interact with host innate and acquired immune cells and humoral mediators accounts for much of the clinical threat associated with Gram-negative infections.

LPS – structure and activity

LPS molecules are amphiphillic, covalent constructs of lipids and polysaccharide and are essential for bacterial growth and viability. A single bacterial cell contains approximately 3.5×10^6 LPS molecules which cover approximately 75% of the bacterial surface [10]. As shown in Figure 1, the LPS molecule consists of three different parts: an outer polysaccharide or "O-specific chain", a core oligosaccharide and lipid A. Integrity of membrane architecture and its LPS component is essential for bacterial viability [11]. In fact, mutants that are unable to synthesize LPS are not viable.

The minimal LPS structure required for growth of the bacterium consists of the lipid A part and at least 1 KDO domain [12,13]. Although not required for growth in the laboratory, additional O-specific chain sugars may be present which help bacteria to resist antibiotics, the complement system and other environmental stresses [12,14-16]. The O-specific chain is characteristic for a given LPS and its bacterial origin and therefore an important virulence factor [10]. It is also responsible for the appearance of the LPS. Colonies of bacteria whose LPS molecules lack this chain have a rough appearance when plated and their LPS is therefore referred to as R(ough)-LPS. Bacteria whose LPS molecules contain the chain have a smooth appearance when grown on plates. Therefore LPS from these bacteria is called S(mooth)-LPS. The lipid A part, which is the chemically most uniform part, is the actual toxic moiety of the LPS molecule. When chemically synthesized lipid A is injected into animals, a similar spectrum of effects is observed as when the whole LPS molecule is injected [17]. Lipid A contains two phosphate groups essential for its biological activity. Lipid A containing only one phosphate group, monophosphoryl lipid A (MPLA), is essentially non-toxic and even able to attenuate the lethal effects of LPS [18]. Full endotoxic activity is expressed when the LPS molecule contains two hexosamine residues, two phosphoryl groups and six fatty acids with a defined chain length and in a distinct location [19,20].

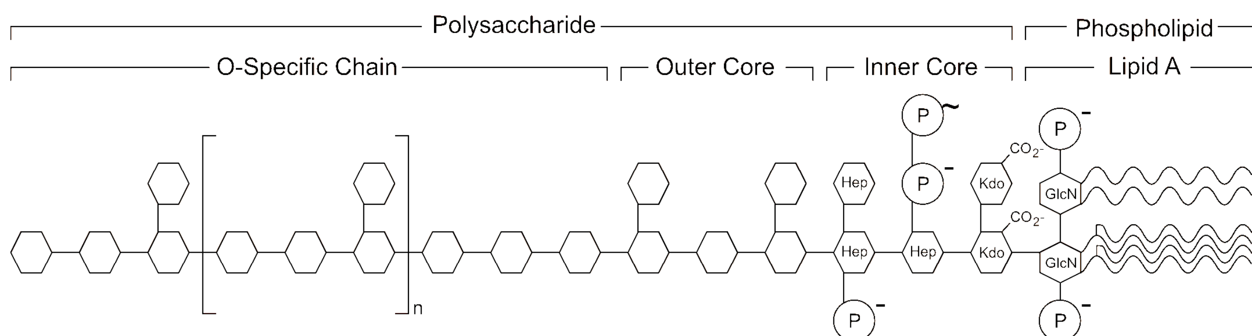

Figure 1: Chemical structure of LPS

LPS binding – receptors and signal transduction

LPS normally remains associated with living bacteria but is spontaneously released during normal bacterial growth and during lysis and death of the bacterium [21]. The LPS molecule is not toxic when incorporated in the bacterial outer membrane. After release from the bacterial wall, its toxic moiety, lipid A, is exposed to immune cells, thereby evoking an inflammatory response. The LPS molecule itself is not intrinsically toxic, it mediates its toxic effects through interactions with receptors on the surface of host endothelial cells, polymorphonuclear leucocytes and macrophages/monocytes which results in the production and release of many inflammatory mediators [22]. When present in the systemic circulation, LPS can associate with several serum components like albumin, transferrin, lactoferrin, hemoglobin, lysozyme and a 28 kDa mannose-binding protein [23]. Other circulating LPS-binders include HDL and LDL, BPI and the cationic proteins CAP18, CAP37 and P15A/P15B, all of which have LPS-neutralizing capacities [24-30]. However, the most important serum protein with regard to endotoxicity is the LPS-binding protein (LBP).

<u>LBP</u>

LBP was first isolated from rabbit acute-phase serum [31]. It is a glycosylated 58-60.5 kDa acute phase protein which is synthesised in the hepatocytes of the liver in response to IL-1 and IL-6 and then secreted into the circulation [32,33]. Besides in the liver, LBP is also produced in the lungs, kidneys and the heart [34]. In normal serum, LBP levels are low (14-22 µg/ml) but can increase up to 200 µg/ml during acute phase [35,36]. The biological activity of LBP is based on its capacity to associate with LPS, reduce LPS aggregates to monomers and transfer these to the cellular binding sites (i.e. CD14), thereby enhancing the LPS-induced activation of monocytes, macrophages and PMN by 100- to 1000-fold [37]. LBP may thus be regarded as a biological amplifier enabling the host to detect small LPS amounts that signal the invasion of Gram-negative bacteria.

<u>CD14</u>

A structure on the surface of LPS-responsive cells that is important for both LPS binding and LPS-induced cellular activation is membrane-bound CD14 (mCD14). It is a 53 kDa GPI-anchored glycoprotein present on the surface of myeloid cells, including monocytes, macrophages, B cells, liver parenchymal cells, gingival fibroblasts and microglial cells [22,38]. Its significance as an LPS receptor was recognized when it was observed that CD14 serves as a LPS binding site when LPS is complexed to LBP [39]. LPS binding to CD14 results in internalization of the CD14 molecule, followed by cell activation and, after 15-30 minutes, the production of cytokines and adhesion molecules [40]. When high concentrations of LPS are present, LBP/CD14-independent cell activation can occur.

CD14 also exists in soluble form (sCD14), which occurs in the circulation at a concentration of 2-6 μg/ml [41]. Several types can be present, ranging from 48 to 55 kDa, which results from either shedding of mCD14 or from GPI-free production [42,43]. sCD14 is able to directly interact with LPS [44]. Here, LBP seems to play only a catalytic role, it does not paticipate in complex formation [45]. The sCD14/LPS complex can bind to CD14-negative cells (i.e. endothelial and epithelial cells) and activate them to produce inflammatory mediators [41,46]. Since the GPI-anchor of mCD14 does not allow direct signal transduction, and sCD14/LPS complexes can activate CD14-negative cells, the presence of an additional molecule involved in LPS binding and signalling was expected.

Toll like receptor (TLR)

In 1996, Toll was shown to be an essential receptor for host defence against fungal infection in *Drosophila* [47]. Not much later a mammalian homolog of the Toll receptor (now called TLR4) was identified and characterized as the LPS-receptor [48-50], a function that was supported by studies in which the defective gene in LPS-unresponsive mouse strains was cloned [48,51,52]. It is now known that, besides TLR4, also TLR2 can function as receptor for LPS [53]. Up till now, at least 11 TLRs have been identified with a wide range of ligand specificity including bacterial, fungal, yeast, protozoan and viral components [54,55].

The TLRs represent a family of type I transmembrane proteins consisting of an extracellular leucine-rich repeat region and a cytoplasmic domaine homologous to that of IL-1 receptors [55,56]. TLRs are expressed in a variety of cell types, predominantly in the cells of the immune system, including macrophages and dendritic cells (DCs), and control the activation of these cells [57,58]. During infection, upregulation of TLRs occurs on cells normally expressing very low levels [59]. The extracellular MD-2 molecule is a polypeptide monomer of 18.4 kDa closely associated with TLR4 and required for its activation [60,61]. For instance, MD-2 knockout mice do not respond to LPS and survive endotoxic shock whereas cells that have downregulated MD-2 and/or TLR4 expression show impaired LPS signalling [62]. In addition, although MD-2 can directly bind LPS, crosslinking of TLR4 and MD2 results in a 20 times higher LPS affinity.

Recognition of LPS by TLR4 initiates either a MyD88-dependent pathway or a MyD88-independent pathway [55,63,64]. As a result, NFκB transcription factors and members of the MAP kinase family are activated (see Figure 2), followed by the production and secretion of reactive oxygen species and inflammatory cytokines and expression of costimulatory molecules and adhesion molecules.

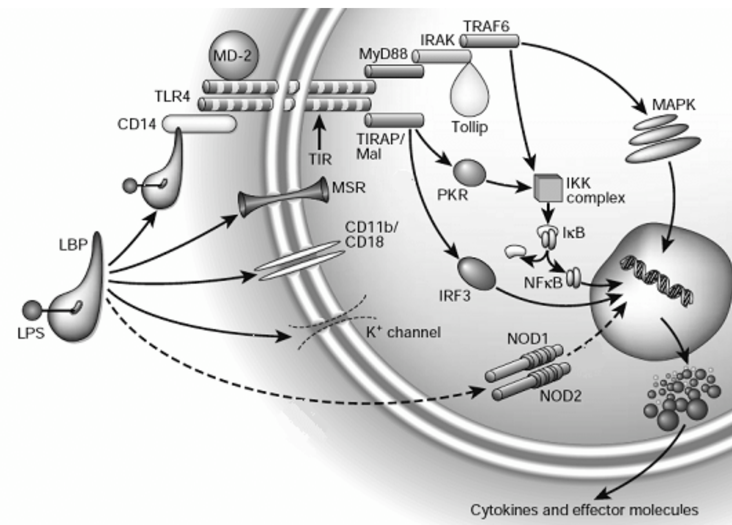

Figure 2: Cell surface recognition of lipopolysaccharide (LPS)

The principal mechanism by which LPS is sensed is via an LPS-binding protein (LBP)-LPS complex and then signalling through the Toll-like receptor 4 (TLR4)-MD2 complex. However, other cell surface molecules also sense LPS, these include the macrophage scavenger receptor (MSR), CD11b/CD18 and ion channels. Picture adapted from Cohen [6]

LPS-induced cell responses

Under physiological conditions immune cells are continuously exposed to low levels of LPS derived from gastrointestinal bacteria. This LPS, essential to maintain a basal level of attentiveness of the immune system, is removed from the blood by monocytes and macrophages. The presence of higher levels of LPS in the circulation activates macrophages and other host cells. This is followed by the production of many inflammatory mediators, including cytokines, chemokines and reactive oxygen species. TNFα and IL-1 induce expression of tissue factor and downregulation of thrombomodulin, which reduces the anticoagulant properties of the endothelial surface and enhances its procoagulant activity [65-67]. Both TNFα and IL-1 promote expression of adhesion molecules on endothelial cells such as ICAM-1, ELAM-1 and VCAM-1, which mediate the adherence of leukocytes to the endothelium [68-70]. These cytokines also directly exert their toxic effects on endothelial cells and enhance permeability [71-73]. Interaction of TNFα and IL-1 with endothelial cells results in the production of IL-1, IL-6, IL-8, MCP-1, PAF and the prostaglandins E_2 and I_2 as well as the generation of vasodilating substances via induction of NO synthase [74-77]. These mediators all contribute to profound microcirculatory effects.

Damage to the endothelial linings by inflammation as well as the expression of tissue factor triggers the coagulation cascade, which results in the formation of blood clots. Normally, the formation of blood clots is controlled by fibrinolysis, but during sepsis fibrinolysis is suppressed [78]. In this situation, blood flow to vital organs and extremities becomes blocked, thereby causing disseminated intravascular coagulation (DIC). If the inflammatory process continues, severe sepsis can occur, leading to decreased blood flow to vital organs (hypoperfusion), tissue hypoxia, multiple organ failure and even death [5,7].

In addition, LPS can activate the complement system and result in the generation of the anaphylatoxins C3a, C4a and C5a, which are responsible for vasodilation, increased vascular permeability, platelet aggregation, and activation and aggregation of granulocytes [79-81]. An important effect of complement stimulation is the activation of neutrophils. Activated neutrophils become adherent to each other and to the endothelium, resulting in the production of arachidonic acid metabolites (leukotrienes, prostaglandins), oxygen radicals and lysosomal enzymes [82]. The interaction of complement with neutrophils, platelets, mastcells and monocytes also stimulates the release of other substances like histamine, serotonin, PAF, and cytokines [83,84]. These mediators produce additional vasoactive effects resulting in capillary leak, vasodilation and microvascular permeability with leakage of plasma components and extravasation of (activated) leukocytes to tissues and organs. An overview of the LPS-induced cell responses is given in Figure 3.

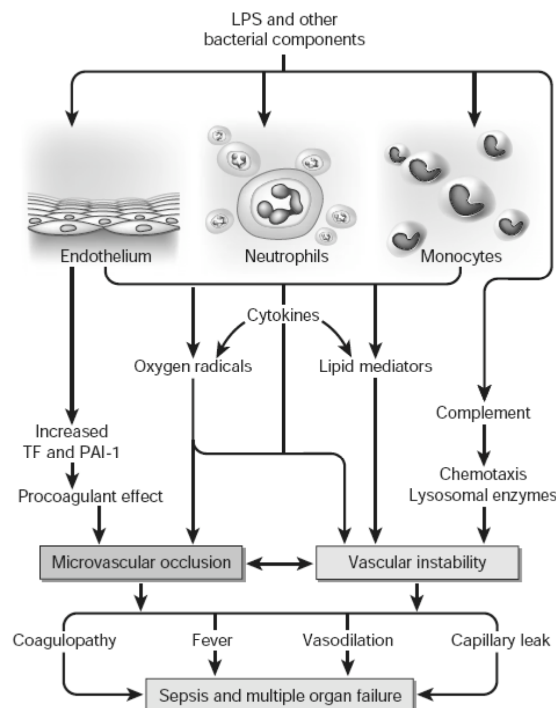

Figure 3: Pathogenetic networks in shock

Lipopolysaccharide and other microbial components simultaneously activate multiple parallel cascades that contribute to the pathophysiology of shock. Picture from Cohen [6].

The cytokine network

Cytokines serve a beneficial role in the immune defence against microorganisms and are necessary to combat localized infections [85]. At homeostasis cytokines are usually absent from the plasma but when high levels of LPS are present such large amounts of pro- as well as anti-inflammatory cytokines are produced that they can be detected in the circulation and produce the features of sepsis [85]. Cytokines can act pro- or anti-inflammatory. The principal pro-inflammatory cytokines are TNFα, IL-1 β, Il-6 and IL-8. For some cytokines it has been demonstrated that circulating levels correspond to severity of sepsis and may predict outcome [86]. The plasma concentration of cytokines varies greatly in patients with sepsis [85]. In general, pro-inflammatory cytokines can be detected in only a small subset of patients whereas anti-inflammatory cytokines and soluble inhibitors can be seen in virtually all patients with sepsis. Despite the fact that TNFα and IL-1 β are not always found in the plasma of septic patients, extensive animal experiments, observations in human septic shock patients and studies in human volunteers all pointed to TNFα and IL-1β as the major inducers of septic shock [87]. The biological effects of pro-inflammatory cytokines are balanced by several anti-inflammatory cytokines, like IL-10, soluble receptors for TNFα and IL-1β, and IL-1 receptor antagonist (IL-1ra), which prevent homeostatic dysfunction and return the system to normal [88].

LPS-induced inflammatory mediators

TNFα

TNFα. is an early marker of sepsis that is produced by monocytes and macrophages in response to various stimuli, including LPS. It exerts a wide variety of effects including hemodynamic instability, fever, diarrhea, metabolic acidosis, capillary leak, activation of coagulation, neurotoxicity and renal- and hematological disorders [89]. At low concentrations, it upregulates vascular adhesion molecules, activates neutrophils, and stimulates monocytes to secrete IL-1, IL-6 and more TNFα. At higher concentrations it acts by further stimulating cytokine liberation from mononuclear cells, activating the coagulation system, and suppressing bone marrow stem cell maturation. At even higher concentrations, TNFα has many deleterious effects, including myocardial depression, hypotension (probably through induction of nitric oxide synthesis) and induction of disseminated intravascular coagulation (DIC).

IL-1β

A variety of cells, amongst which activated mononuclear cells, produce IL-1 β in response to LPS and other stimuli [90]. Injection of low IL-1 β doses into animals results in hypotension, increased cardiac output and heart rate, leukopenia, thrombocytopenia, haemorrhage and pulmonary edema [91]. The endocrine effects of high IL-1β doses are similar to TNFα, causing fever and hypotension [90].

IL-6

A cytokine often found in septic patients is IL-6. Levels of circulating IL-6 correlate with severity of sepsis and may predict outcome [92]. IL-6 is produced by macrophages upon stimulation with IL-1 or TNFα. In order to propagate the inflammatory process, IL-6 can act as a pro-inflammatory cytokine on hepatocytes and B cells. Upon IL-6 stimulation, hepatocytes secrete increased levels of acute phase proteins [93]. On B-cells IL-6 acts as a growth factor, thereby promoting antibody formation and release. In contrast to its pro-inflammatory capacities, IL-6 can also act as an anti-inflammatory cytokine by inducing the release of IL-1ra and sTNFR [93].

IL-8

The chemokine IL-8 can also be detected in the plasma during sepsis. It is produced by a variety of cells, including macrophages, monocytes, neutrophils and endothelial cells. It exists not only as a free chemokine but also as a cell-associated form [94,95]. IL-8 has chemoattractant activity, is able to induce degranulation, and to elicit a respiratory burst [96,97]. In addition, it may promote adherence of neutrophils to endothelium [98]. In contrast, under certain conditions this chemokine can also inhibit this process [99].

NO

LPS, TNFα, IL-1β and IFNγ are known to up regulate the expression of inducible nitric oxide synthase (iNOS) in the endothelium, vascular smooth muscle cells, macrophages and different parenchymal cells, which leads to increased nitric oxide (NO) production [100]. NO can serve pro- as well as anti-inflammatory functions. NO is important for cardiovascular homeostasis by regulating blood pressure and blood flow distribution. The molecule is also important in host defence since it inhibits the growth of many bacteria and parasites [101]. Overexpression of NO, however, may result in tissue injury, multiple organ dysfunction and death [102]. NO therefore is an important mediator in the inflammatory responses during sepsis and septic shock [103].

Anti-sepsis therapies

Early recognition of sepsis and prompt treatment are essential for patient survival. The standard treatment of bacterial sepsis often consists of administration of fluid and vasopressors to restore blood pressure, organ flow and tissue perfusion and administration of antibiotics [104-106]. During the last two decades a significant amount of anti-sepsis therapies, each of them directed at different mediators produced during sepsis, have been explored [107]. An overview of clinical sepsis trials is given in Figure 4.

Target	Intervention
Immune response	Glucocorticoids
LPS	Anti-endotoxin antibodies LPS neutralization (BPI) LPS elimination (hemofiltration) LPS binding by HDL
Lipid A	Lipid A analogs E5531 and lipid IVa Deacylated lipid A
Cytokines	TNFα antibodies Soluble TNF receptor IL-1 receptor antagonist
Nitric oxide	NOS inhibitor (L-NAME) INOS inhibitor (L-NMMA) Guanylyl cyclase inhibitor (methylene blue) NO scavenger (PHP)
Lipid mediators	Thromboxane A2 inhibitor (Dazoxiben) Thromboxane synthase inhibitor (Ketoconazole) Cyclooxygenase inhibitors (Ibuprofen) PAF antagonist Bradykinin receptor antagonist
Coagulation/inflammation	Inhibition thrombin, Factors Ixa, Xa and XIA, XIIa (AT-III) Inhibition factors X and IX (TFPI) Inactivation factors Va and VIIIa (APC) *

Figure 4: Clinical trials in sepsis

Various strategies in clinical trials are grouped according to a common target.

* Approved for treatment in septic patients. Information adapted from Riedemann *et al.* [107]

Since TNFα and IL-1β appear to be the earliest mediators of the subsequent host response [108-110], many anti-sepsis therapies focused on neutralizing or antagonizing these two cytokines [85]. Other therapies aimed at inhibiting NO-synthesis or using anticoagulantia [85,108]. Recently, the anticoagulant and anti-inflammatory agent Activated Protein C (Xigris) was reported to reduce overall mortality in severe sepsis patients with 6% [111]. At present, Xigris is recommended only for adult patients who are at high risk of death from severe sepsis.

The successes obtained with animal models were, however, not necessarily reproduced in patient trials. Many experimental animal models are based on a bolus injection of LPS or bacteria and may

therefore not resemble the conditions in patients. In addition, the time courses of cytokine release in animal models are usually very different from those in patients. In fact, cytokines and other mediators are in principle beneficial to the patient, as they are needed to fight infections. Inhibiting these mediators may therefore be deleterious to the patient. Finally, inhibiting one mediator might not be successful since other mediators and their effects are still present. The best anti-sepsis therapy therefore seems to be an intervention that inhibits or neutralizes the initiator of the sepsis cascade: LPS. However, the therapies that explored LPS neutralization by using anti-LPS antibodies, LPS 'binding' proteins or scavenging by HDL particles did not give any improvement [112].

Alkaline phosphatase and LPS

Like deacylated LPS, dephosphorylated LPS appears to have a decreased biological activity [113]. Despite this knowledge, no attempts have been made to enzymatically dephosphorylate, and thereby detoxify, LPS until the late 90's.

In 1997, Poelstra *et al.* [114] showed that human placental alkaline phosphatase (HPLAP) dephosphorylates and thereby detoxifies LPS at physiological pH levels. By measuring the release of inorganic phosphate (Pi), this study clearly demonstrated the dephosphorylation of LPS by AP. Later it was shown that HPLAP reduces mortality in mice lethally infected with Gram-negative bacteria, thereby suggesting a role for alkaline phosphatase in protection against Gram-negative sepsis [115,116].

Alkaline phosphatase is expressed in various tissues throughout the body and has been shown to have a dual mode of action. First, it dephosphorylates LPS, thereby eliminating the root cause of the sepsis cascade. Secondly, the enzymatic reaction product dephosphorylated LPS can act as antagonist on the LPS receptor complex. Figure 5 shows that alkaline phosphatase interferes with the sepsis cascade at two distinc sites, each of which contributes to inhibition of the deleterious downstream effects of LPS.

AP is excessively expressed in the canalicular membranes of hepatocytes after exposure to LPS [117,118]. Since AP is also present at various strategic positions in the body (intestine, kidneys, and liver) and it's action being at the front end of the inflammatory cascade, AP may be part of the natural defence system of the body. Consequently, AP potentially encompasses a novel therapeutic in the treatment of Gram-negative sepsis and other LPS-mediated diseases.

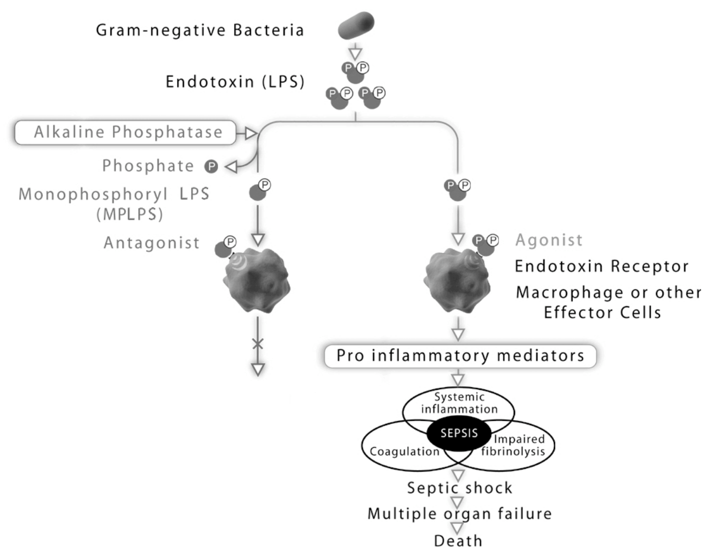

Figure 5: Alkaline phosphatase's dual mode of action
Source: AM-Pharma, Bunnik

Alkaline phosphatase

Alkaline phosphatases (APs) catalyze the hydrolysis of phosphomonoesters with release of inorganic phosphate at alkaline pH *in vitro* [119]. It is an endogenous enzyme attached to the cell membrane via a glycosylphosphatidylinositol (GPI)-anchor, making it an ectoenzyme capable of enzymatic activity outside the cell. A soluble form of AP is found in plasma, probably due to the action of phospholipases that release it from the cell membrane. It is a dimeric metalloenzyme containing two Zn^{2+} and one Mg^{2+} in each active site region, which are essential for its enzymatic activity. Zn^{2+} is known to keep the enzyme in the required conformation for optimal catalytic activity whereas Mg^{2+} is needed for full catalytic activity.

The enzyme is common to all organisms and can be found in many tissues throughout the body. In mammals, this group of enzymes can be classified as either tissue-specific, which include placental (PLAP), germ cell (GCAP) and intestinal (IAP) isoenzymes, or tissue non-specific (TNAP), which includes the bone-liver-kidney (BLK) isotype [120]. The tissue-specific isoforms are 90-98% homologous and their genes are clustered on chromosome 2. The TNAP gene is located on chromosome 1 and the iso-enzyme is about 50% homologous to the other three. The structure and amino-acid sequence of the human isoforms have been fully described [121]. The different isoforms serve different biological fuctions. For example, bone mineralization and vitamin B6 utilization (TNAP) [122,123], maternal-fetal interactions [124-127] and capture of circulating IgG (PLAP) [128] and transepithelial lipid transport (IAP) [129]. The isoforms also differ in kinetic half-life *in vivo* [130,131]. IAP lacks a terminal sialyc acid group and is therefore recognized by the asialoglycoprotein receptor on hepatocytes [132,133]. This mechanism has been shown to be responsible for the short serum half-life

of IAP. Indeed, it has been shown that the plasma residence time of canine intestinal AP could be extended by blocking the ASGPR [134,135]. PLAP contains sialylated endgroups and therefore has a longer plasma half-life than IAP.

Aim and outline of this thesis

Based on publications showing that alkaline phosphatase (AP) is capable of dephosphorylating LPS and that HPLAP reduces mortality in mice lethally infected with Gram-negative bacteria, it was suggested that AP may be beneficial in Gram-negative sepsis [115,116]. This thesis describes pre-clinical and clinical studies that were performed in order to develop alkaline phosphatase as a novel therapeutic in the treatment of Gram-negative sepsis.

Testing a potential novel therapeutic requires that you have enough of the test compound available to perform all the necessary tests. Bovine (calf) intestinal mucosa represents a rich source of AP (BIAP). Whether BIAP is as effective as HPLAP in detoxifying LPS *in vivo* was studied in **chapter 2**. First, the data obtained with human placental AP were confirmed using BIAP in a mouse model. Second, the LPS-detoxifying capacity of BIAP was studied in a piglet model. In addition, pharmacokinetic and safety studies were performed. The results show that, although BIAP is cleared much faster than HPLAP, it attenuates LPS-mediated toxicity in mice and piglets. Also, long-term administration of high BIAP doses to piglets did not show any signs of toxicity. Therefore, BIAP might potentially encompass a novel therapeutic drug in the treatment of LPS-mediated diseases. But before BIAP can be applied as a novel therapeutic, more data on safety and tolerability had to be generated.

Chapter 3 describes studies in which the biological activity of BIAP was investigated in different *in vitro* systems. Results show that BIAP, when attached to the cell membrane, reduces LPS-induced TNFα, IL-6 and NO_x levels in macrophages and epithelial cells.

Studies on safety and tolerability of BIAP administration are presented in **chapter 4** of this thesis. Since mice and dogs showed great differences in immune reactivity towards the protein, more species were included in the study. In addition, a study on the tolerability and pharmacokinetic behaviour of BIAP in man was performed. The results show that single dosing of BIAP is safe. However, multiple dosing of BIAP to mice resulted in antibody-mediated toxicity whereas dogs, piglets and human showed no side effects.

The last experimental chapter of this thesis (**chapter 5**) investigates if the species differences in immune reactivity towards BIAP observed in chapter 4 are due to a lack of tolerance to bovine proteins. The results show that tolerance induction to BIAP by oral treatment for a prolonged time prevents immunotoxicity of multiple intravenous BIAP administration to mice, which was read by the inability to mount a BIAP-specific antibody response.

References

1 Angus DC, Linde-Zwirble WT, Lidicker J, et al. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 2001; 29:1303-1310

2 Riedemann NC, Guo RF, Ward PA. The enigma of sepsis. J Clin Invest 2003; 112:460-467

3 Vincent JL, Carlet J, Opal SM. In: The sepsis text. Boston/Dorderecht/London: Kluwer Academic Publishers, 2002

4 Scheinkestel CD, Davies AR, Bristow PJ. Evidence-based medicine in intensive care. Med J Aust 2001; 174:526-527

5 Bone R. In: sepsis and multiorgan failure: Williams and Wilkins, 1997

6 Cohen J. The immunopathogenesis of sepsis. Nature 2002; 420:885-891

7 Emory University Health Sciences Center. Sepsis on the increase in U.S., according to Emory University and CDC study; Available at: www.eurekalert.org/pub_releases/2002-2005/euhs-so051502.php. Accessed June 051521, 052005

8 Rangel-Frausto. In: Sepsis and multiorgan failure: Williams and Wilkins, 1997

9 Bochud PY, Bonten M, Marchetti O, et al. Antimicrobial therapy for patients with severe sepsis and septic shock: an evidence-based review. Crit Care Med 2004; 32:S495-512

10 Rietschel ET, Kirikae T, Schade FU, et al. Bacterial endotoxin: molecular relationships of structure to activity and function. Faseb J 1994; 8:217-225

11 Galloway SM, Raetz CR. A mutant of Escherichia coli defective in the first step of endotoxin biosynthesis. J Biol Chem 1990; 265:6394-6402

12 Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol Rev 1993; 57:655-682

13 Brabetz W, Muller-Loennies S, Holst O, et al. Deletion of the heptosyltransferase genes rfaC and rfaF in Escherichia coli K-12 results in an Re-type lipopolysaccharide with a high degree of 2-aminoethanol phosphate substitution. Eur J Biochem 1997; 247:716-724

14 Reeves PR, Hobbs M, Valvano MA, et al. Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol 1996; 4:495-503

15 Whitfield C, Amor PA, Koplin R. Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. Mol Microbiol 1997; 23:629-638

16 Whitfield C. Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol 1995; 3:178-185

17 Galanos C, Luderitz O, Rietschel ET, et al. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. Eur J Biochem 1985; 148:1-5

18 Chase JJ, Kubey W, Dulek MH, et al. Effect of monophosphoryl lipid A on host resistance to bacterial infection. Infect Immun 1986; 53:711-712

19 Loppnow H, Brade H, Durrbaum I, et al. IL-1 induction-capacity of defined lipopolysaccharide partial structures. J Immunol 1989; 142:3229-3238

20 Golenbock DT, Hampton RY, Qureshi N, et al. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J Biol Chem 1991; 266:19490-19498

21 Hellman J, Loiselle PM, Tehan MM, et al. Outer membrane protein A, peptidoglycan-associated lipoprotein, and murein lipoprotein are released by Escherichia coli bacteria into serum. Infect Immun 2000; 68:2566-2572
22 Rietschel ET, Wagner H. Pathology of septic shock: Springer-Verlag Berlin Heidelberg, 1996
23 Morrison D. Diversity of mammalian macromolecules which bind to bacterial lipopolysaccharides. Excerpta Med Int Congr Ser 1990; 923:183-189
24 Ulevitch RJ, Johnston AR, Weinstein DB. New function for high density lipoproteins. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. J Clin Invest 1981; 67:827-837
25 Munford RS, Hall CL, Lipton JM, et al. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of Salmonella typhimurium lipopolysaccharides. J Clin Invest 1982; 70:877-888
26 Flegel WA, Wolpl A, Mannel DN, et al. Inhibition of endotoxin-induced activation of human monocytes by human lipoproteins. Infect Immun 1989; 57:2237-2245
27 Weiss J, Elsbach P, Shu C, et al. Human bactericidal/permeability-increasing protein and a recombinant NH2-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. J Clin Invest 1992; 90:1122-1130
28 Marra MN, Wilde CG, Collins MS, et al. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. J Immunol 1992; 148:532-537
29 Levy O, Weiss J, Zarember K, et al. Antibacterial 15-kDa protein isoforms (p15s) are members of a novel family of leukocyte proteins. J Biol Chem 1993; 268:6058-6063
30 Larrick JW, Morgan JG, Palings I, et al. Complementary DNA sequence of rabbit CAP18--a unique lipopolysaccharide binding protein. Biochem Biophys Res Commun 1991; 179:170-175
31 Tobias PS, Soldau K, Ulevitch RJ. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J Exp Med 1986; 164:777-793
32 Schumann RR, Kirschning CJ, Unbehaun A, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. Mol Cell Biol 1996; 16:3490-3503
33 Schumann RR, Zweigner J. A novel acute-phase marker: lipopolysaccharide binding protein (LBP). Clin Chem Lab Med 1999; 37:271-274
34 Su GL, Freeswick PD, Geller DA, et al. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence for extrahepatic expression. J Immunol 1994; 153:743-752
35 Tobias PS, Ulevitch RJ. Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. Immunobiology 1993; 187:227-232
36 Gallay P, Heumann D, Le Roy D, et al. Mode of action of anti-lipopolysaccharide-binding protein antibodies for prevention of endotoxemic shock in mice. Proc Natl Acad Sci U S A 1994; 91:7922-7926
37 Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. Science 1990; 249:1429-1431

38 Van Amersfoort ES, Van Berkel TJ, Kuiper J. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev 2003; 16:379-414
39 Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249:1431-1433
40 Detmers PA, Thieblemont N, Vasselon T, et al. Potential role of membrane internalization and vesicle fusion in adhesion of neutrophils in response to lipopolysaccharide and TNF. J Immunol 1996; 157:5589-5596
41 Frey EA, Miller DS, Jahr TG, et al. Soluble CD14 participates in the response of cells to lipopolysaccharide. J Exp Med 1992; 176:1665-1671
42 Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991; 147:1567-1574
43 Durieux JJ, Vita N, Popescu O, et al. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. Eur J Immunol 1994; 24:2006-2012
44 Hailman E, Lichenstein HS, Wurfel MM, et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 1994; 179:269-277
45 Tobias PS, Soldau K, Gegner JA, et al. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. J Biol Chem 1995; 270:10482-10488
46 Pugin J, Schurer-Maly CC, Leturcq D, et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A 1993; 90:2744-2748
47 Lemaitre B, Nicolas E, Michaut L, et al. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996; 86:973-983
48 Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282:2085-2088
49 Chow JC, Young DW, Golenbock DT, et al. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem 1999; 274:10689-10692
50 Lien E, Means TK, Heine H, et al. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest 2000; 105:497-504
51 Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999; 162:3749-3752
52 Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 1999; 189:615-625
53 Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature 1998; 395:284-288
54 Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005; 17:1-14
55 Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4:499-511
56 Rock FL, Hardiman G, Timans JC, et al. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 1998; 95:588-593

57 Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001; 1:135-145
58 Muzio M, Mantovani A. Toll-like receptors (TLRs) signalling and expression pattern. J Endotoxin Res 2001; 7:297-300
59 Bosisio D, Polentarutti N, Sironi M, et al. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood 2002; 99:3427-3431
60 Shimazu R, Akashi S, Ogata H, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999; 189:1777-1782
61 Nagai Y, Akashi S, Nagafuku M, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol 2002; 3:667-672
62 Abreu MT, Vora P, Faure E, et al. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 2001; 167:1609-1616
63 Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004; 16:3-9
64 Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001; 2:675-680
65 Bevilacqua MP, Pober JS, Majeau GR, et al. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci U S A 1986; 83:4533-4537
66 Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 1986; 163:740-745
67 Lentz SR, Tsiang M, Sadler JE. Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. Blood 1991; 77:542-550
68 Gamble JR, Harlan JM, Klebanoff SJ, et al. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci U S A 1985; 82:8667-8671
69 Mantovani A, Dejana E. Cytokines as communication signals between leukocytes and endothelial cells. Immunol Today 1989; 10:370-375
70 Briscoe DM, Cotran RS, Pober JS. Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration. J Immunol 1992; 149:2954-2960
71 Martin S, Maruta K, Burkart V, et al. IL-1 and IFN-gamma increase vascular permeability. Immunology 1988; 64:301-305
72 Schuger L, Varani J, Marks RM, et al. Cytotoxicity of tumor necrosis factor-alpha for human umbilical vein endothelial cells. Lab Invest 1989; 61:62-68
73 Robaye B, Mosselmans R, Fiers W, et al. Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. Am J Pathol 1991; 138:447-453
74 Kawakami M, Ishibashi S, Ogawa H, et al. Cachectin/TNF as well as interleukin-1 induces prostacyclin synthesis in cultured vascular endothelial cells. Biochem Biophys Res Commun 1986; 141:482-487

75 Nawroth PP, Bank I, Handley D, et al. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 1986; 163:1363-1375
76 Sica A, Matsushima K, Van Damme J, et al. IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. Immunology 1990; 69:548-553
77 Sica A, Wang JM, Colotta F, et al. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. J Immunol 1990; 144:3034-3038
78 Hesselvik JF, Blomback M, Brodin B, et al. Coagulation, fibrinolysis, and kallikrein systems in sepsis: relation to outcome. Crit Care Med 1989; 17:724-733
79 Bjork J, Hugli TE, Smedegard G. Microvascular effects of anaphylatoxins C3a and C5a. J Immunol 1985; 134:1115-1119
80 Williams TJ. Vascular permeability changes induced by complement-derived peptides. Agents Actions 1983; 13:451-455
81 Tanaka T, Abe M, Mitsuyama T, et al. Hyperresponsiveness of granulocytes to anaphylatoxins, C5a and C3a, in Churg-Strauss syndrome. Intern Med 1995; 34:1005-1008
82 Roos D. Neutrofielen en ontsteking. Ned. Tijdschr Klin Chem 1995; 20:76-81
83 Cavaillon JM, Fitting C, Haeffner-Cavaillon N. Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. Eur J Immunol 1990; 20:253-257
84 Scholz W, McClurg MR, Cardenas GJ, et al. C5a-mediated release of interleukin 6 by human monocytes. Clin Immunol Immunopathol 1990; 57:297-307
85 van der Poll T. Immunotherapy of sepsis. Lancet Infect Dis 2001; 1:165-174
86 Bozza FA, Bozza PT, Castro Faria Neto HC. Beyond sepsis pathophysiology with cytokines: what is their value as biomarkers for disease severity? Mem Inst Oswaldo Cruz 2005; 100 Suppl 1:217-221
87 Minnich DJ, Moldawer LL. Anti-cytokine and anti-inflammatory therapies for the treatment of severe sepsis: progress and pitfalls. Proc Nutr Soc 2004; 63:437-441
88 Thijs LG, Hack CE. Time course of cytokine levels in sepsis. Intensive Care Med 1995; 21 Suppl 2:S258-263
89 Van der Poll T, Romijn JA, Endert E, et al. Tumor necrosis factor mimics the metabolic response to acute infection in healthy humans. Am J Physiol 1991; 261:E457-465
90 Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood 1991; 77:1627-1652
91 Okusawa S, Gelfand JA, Ikejima T, et al. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J Clin Invest 1988; 81:1162-1172
92 Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. Ann Intern Med 1993; 119:771-778
93 Tilg H, Trehu E, Atkins MB, et al. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. Blood 1994; 83:113-118
94 Marty C, Misset B, Tamion F, et al. Circulating interleukin-8 concentrations in patients with multiple organ failure of septic and nonseptic origin. Crit Care Med 1994; 22:673-679

95 Marie C, Fitting C, Cheval C, et al. Presence of high levels of leukocyte-associated interleukin-8 upon cell activation and in patients with sepsis syndrome. Infect Immun 1997; 65:865-871
96 Collins PD, Jose PJ, Williams TJ. The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationship between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein 1. J Immunol 1991; 146:677-684
97 Schroder JM. The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not the release of cellular arachidonate. J Exp Med 1989; 170:847-863
98 Huber AR, Kunkel SL, Todd RF, 3rd, et al. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 1991; 254:99-102
99 Gimbrone MA, Jr., Obin MS, Brock AF, et al. Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. Science 1989; 246:1601-1603
100 Beck KF, Eberhardt W, Frank S, et al. Inducible NO synthase: role in cellular signalling. J Exp Biol 1999; 202:645-653
101 De Groote MA, Fang FC. NO inhibitions: antimicrobial properties of nitric oxide. Clin Infect Dis 1995; 21 Suppl 2:S162-165
102 Burgner D, Rockett K, Kwiatkowski D. Nitric oxide and infectious diseases. Arch Dis Child 1999; 81:185-188
103 Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis--an overview. Acta Anaesthesiol Scand 1999; 43:275-288
104 Cohen J, Glauser MP. Septic shock: treatment. Lancet 1991; 338:736-739
105 Rackow EC, Astiz ME. Pathophysiology and treatment of septic shock. Jama 1991; 266:548-554
106 Wheeler AP, Bernard GR. Treating patients with severe sepsis. N Engl J Med 1999; 340:207-214
107 Riedemann NC, Guo RF, Ward PA. Novel strategies for the treatment of sepsis. Nat Med 2003; 9:517-524
108 Howe LM. Novel agents in the therapy of endotoxic shock. Expert Opin Investig Drugs 2000; 9:1363-1372
109 Lin E, Lowry SF. The human response to endotoxin. Sepsis 1998; 2:255-262
110 Baumann H, Gauldie J. The acute phase response. Immunol Today 1994; 15:74-80
111 Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001; 344:699-709
112 Vesy CJ, Kitchens RL, Wolfbauer G, et al. Lipopolysaccharide-binding protein and phospholipid transfer protein release lipopolysaccharides from gram-negative bacterial membranes. Infect Immun 2000; 68:2410-2417
113 Dziarski R. Peptidoglycan and lipopolysaccharide bind to the same binding site on lymphocytes. J Biol Chem 1991; 266:4719-4725
114 Poelstra K, Bakker WW, Klok PA, et al. A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Invest 1997; 76:319-327
115 Poelstra K, Bakker WW, Klok PA, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

116 Bentala H, Verweij WR, Huizinga-Van der Vlag A, et al. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566

117 Xu Q, Lu Z, Zhang X. A novel role of alkaline phosphatase in protection from immunological liver injury in mice. Liver 2002; 22:8-14

118 Saetre T, Hovig T, Roger M, et al. Hepatocellular damage in porcine endotoxemia: beneficial effects of selective versus non-selective nitric oxide synthase inhibition? Scand J Clin Lab Invest 2001; 61:503-512

119 McComb RB, Bowers GN, Posen S. Alkaline phosphatases. New York: Plenum Press, 1979

120 Fishman WH. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

121 Le Du MH, Stigbrand T, Taussig MJ, et al. Crystal structure of alkaline phosphatase from human placenta at 1.8 A resolution. Implication for a substrate specificity. J Biol Chem 2001; 276:9158-9165

122 Mornet E, Stura E, Lia-Baldini AS, et al. Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization. J Biol Chem 2001; 276:31171-31178

123 Narisawa S, Wennberg C, Millan JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. J Pathol 2001; 193:125-133

124 Beckman G, Beckman L, Holm S, et al. Placental alkaline phosphatase types and transplacental IgG transport. Hum Hered 1995; 45:1-5

125 Beckman G, Beckman L, Magnusson SS. Placental alkaline phosphatase phenotypes and pre-natal selection. Evidence from studies of spontaneous and induced abortions. Hum Hered 1972; 22:473-480

126 Beckman L, Beckman G, Mi MP. The relation between human placental alkaline phosphatase types and some perinatal factors. Hum Hered 1969; 19:258-263

127 Beckman L, Bjorling G, Christodoulou C. Pregnancy enzymes and placental polymorphism. I. Alkaline phosphatse. Acta Genet Stat Med 1966; 16:59-73

128 Van Hoof VO, and De Broe. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. crit. rev. clin. lab. sci. 1994; 31:197-293

129 Mahmood A, Yamagishi F, Eliakim R, et al. A possible role for rat intestinal surfactant-like particles in transepithelial triacylglycerol transport. J Clin Invest 1994; 93:70-80

130 Blom E, Ali MM, Mortensen B, et al. Elimination of alkaline phosphatases from circulation by the galactose receptor. Different isoforms are cleared at various rates. Clin Chim Acta 1998; 270:125-137

131 Posen S. Turnover of circulating enzymes. Clin Chem 1970; 16:71-84

132 Pricer WE, Jr., Ashwell G. The binding of desialylated glycoproteins by plasma membranes of rat liver. J Biol Chem 1971; 246:4825-4833

133 Pricer WE, Jr., Hudgin RL, Ashwell G, et al. A membrane receptor protein for asialoglycoproteins. Methods Enzymol 1974; 34:688-691

134 Scholtens HB, Hardonk MJ, Meijer DK. A kinetic study of hepatic uptake of canine intestinal alkaline phosphatase in the rat. Liver 1982; 2:1-13

135 Scholtens HB, Meijer DK, Hardonk MJ. A histochemical study on the distribution of injected canine intestinal alkaline phosphatase in rat liver. Liver 1982; 2:14-21

2

Bovine calf intestinal alkaline phosphatase, a novel therapeutic drug for 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 the present study it is demonstrated that also alkaline phosphatase derived from calf intestine (BIAP) is able to detoxify LPS. In mice administered BIAP, 80% of the animals survived a lethal *E. coli* infection. In piglets, previous to LPS detoxification, the pharmacokinetic behavior of BIAP was studied. BIAP clearance was shown to be dose-independent and showed a biphasic pattern with an initial $t_{1/2}$ of 3 - 5 minutes and a second phase $t_{1/2}$ of 2 - 3 hours. Although BIAP is cleared much faster than HPLAP, it attenuates LPS-mediated effects on hematology and TNFα responses at doses up to 10 μg/kg in piglets. LPS induced hematological changes were antagonized and the TNFα response was reduced up to 98%. Daily i.v. bolus administration of 4000 units BIAP, the highest dose used in the LPS intervention studies, in piglets for 28 days was tolerated without any sign of toxicity. Therefore, BIAP 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.

Introduction

LPS-mediated diseases comprise a wide variety of acute and chronic diseases, for example sepsis and ulcerative colitis [1,2]. LPS is a constituent of the outer membrane of Gram-negative bacteria and is essential for growth and survival of the bacterium [3]. 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, LDL, HDL and LPS-binding protein (LBP) [4,5]. 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 [6]. The physiological function of signaling through TLRs is to induce the production and release of pro-inflammatory cytokines like TNFα, IL-1, IL-6, IL-8 and IL-12 [7,8]. Also induction of nitric oxide, prostaglandins, leukotrienes and toxic oxygen radicals is reported [9,10].

One of the most life-threatening LPS-mediated diseases, Gram-negative sepsis, is characterized by excessive production of the above mentioned pro-inflammatory cytokines, activation of proteolytic cascades, coagulation abnormalities [11] and hemodynamic responses, resulting in hypotension, poor tissue perfusion and multi-organ failure [9,10]. Since tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) appear to be the earliest mediators of the subsequent host response [12], many anti-sepsis therapies focused on neutralizing or antagonizing these two cytokines [13]. Other therapies aimed at LPS neutralization by using anti-LPS antibodies or LPS 'binding' proteins, scavenging by HDL particles [14], inhibiting NO-synthesis or using anticoagulantia [10,13]. Despite all innovations 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 35-50% [1]. Recently, the anticoagulant and anti-inflammatory agent Activated Protein C is reported to reduce overall mortality in severe sepsis patients with 6% [15].

Poelstra *et al.* [16] and Bentala *et al.* [17] 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 (W. Verweij, personal comm.). AP catalyzes the hydrolysis of phosphomonoesters with release of inorganic phosphate at alkaline pH *in vitro* [18]. In mammals, the AP family consists of several isoenzymes classified in tissue-nonspecific APs (bone-liver-kidney type) and tissue-specific APs (intestinal, placental and germ cell type) [19].

Bovine (calf) intestinal mucosa represents a rich source of IAP (BIAP). Whether this AP also exerts the proposed physiological role *in vivo* is subject of the present study. First, the data obtained with human placental AP [17] were confirmed using BIAP in a mouse model. Here 80% of the BIAP-treated mice survived a lethal gram-negative bacterial infection, likely due to reducing the LPS-mediated inflammatory sepsis cascade. The LPS-detoxifying capacity of BIAP was further

studied in a piglet model. Piglets were treated with LPS ranging from 10 ng to 10 µg/kg bodyweight with or without BIAP and inflammation parameters like TNFα release and hematological parameters were studied. In order to assess the applicability of BIAP as a therapeutic agent, pharmacokinetic and safety studies were performed.

Material and methods

Chemicals

Alkaline phosphatase from calf intestinal mucosa (BIAP) was obtained from Biozyme (Blaenavon, UK), *E. coli* O111:B4 LPS, Glycin and 4-p-nitrophenylphosphate from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), $MgCl_2$ and NaOH from Merck Eurolab BV (Amsterdam, The Netherlands) and heparin (5000 IE/ml) from Leo Pharma BV (Weesp, The Netherlands).

Animals and experimental design

Mice experiments

Specific pathogen free female Balb/c mice (20 gram, from Charles River) were randomly assigned to specific treatment and housed under barrier conditions in filter-topped macrolon cages (5 mice/cage) at the animal housing facility (GDL) with drinking water and standard laboratory food pellets *ad libitum.* At the start, 10 mice received a dose of $2.3x10^7$ colony forming units of freshly cultured *E. coli* bacteria diluted with saline in a 100 µl volume by i.p. injection. BIAP was freshly prepared and 100 µl containing 1.5 units was administered in the tail vein to 5 mice directly after administration of bacteria. Body temperature was measured prior to inoculation of bacteria, and at 1-hour 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 body weight, activity, skin condition and well-being.

Bacterial cell culture

E. coli strain 25922 (ATCC) was cultured for 18 ± 2 hours in BHI 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 $2.3x10^8$ colony forming units/ml and kept on ice until use in order to prevent uncontrolled bacterial growth. 10-Fold dilutions were plated on LB-agar plates and incubated for 18 ± 2 hours at 37˚C to affirm the bacterial concentration administered *in vivo.*

Piglets

Female Dutch Landrace x 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 university of Utrecht (GDL) with a 12 hours light and 12 hours 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 2 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 IE/ml 0,9% NaCl) to prevent blood clotting. During this procedure, animals were sedated with i.m. injection of Ketamine and Stressnil (Azaperone®), followed by induction of anesthesia with an i.v. injection of Thiopental (Nesdonal®) and inhalation of a mixture of O_2 AIR and Fluothane. The catheter to be used for BIAP 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 recuperate for one week during which they received antibiotics (Praxavet and Finedyne). Blood samples were collected every 10 minutes during the first 1,5 hour of the experiments and every hour up till 6 hours after onset of the experiment and used for pharmacokinetic and pharmacodynamic purposes.

Blood sampling

Blood sampling through catheter was carried out under aseptic conditions. Catheter content (dead volume) was removed before blood samples were drawn. The catheter was filled with heparin after each blood sample to prevent blood clotting.

Piglet experiments

In order to obtain i.v. bolus pharmacokinetic data, 400 (n=5) or 4000 (n=6) units BIAP were suspended in 1 ml sterile 0.9% saline solution and administered i.v. as a single shot after which blood samples were drawn. In order to obtain pharmacokinetic data during and after i.v. infusion of BIAP, 2500 (n=5) or 35000 (n=4) units BIAP were suspended in 35 or 40 ml sterile 0.9% saline solution and administered over 50 or 60 minutes with an infusion rate of 0,5 ml per minute, resulting in a final plasma level of 400 or 4000 units per liter respectively.

To study the safety of repeated BIAP administration, piglets were given BIAP each day for 28 consecutive days (n=5). For this purpose, 4000 units of BIAP were suspended in 1 ml sterile 0.9% saline solution and administered as a single i.v. bolus injection. Prior to each BIAP administration blood samples were drawn on which AP activity was determined. Twice a week blood samples were screened for hematological parameters (number of WBC's by auto-analyser, WBC differentiation by May-Grünwald staining and light microscopy and thrombocytes by counting in Bürker chamber by phase-contrast microscopy), liver and/or kidney damage (ureum, creatinin and γ-GT by auto-analyser) and electrolytes (sodium, potassium, chloride, calcium, magnesium and phosphate by auto-analyser). All these tests were performed according to GLP

(good laboratory practice) standards at the clinical hematology facility of the Faculty of Veterinary medicine, Utrecht University (Utrecht, The Netherlands).

Pharmacodynamics of BIAP-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=2) or infusion over 5-10 minutes (n=3). Animals were given BIAP either as a single i.v. bolus prior to LPS administration (n=4) or as an infusion during LPS administration (n=8). Animals were sacrificed 24-hours 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 minutes, 15˚C) after which the plasma was harvested and AP activity was determined. In brief, 5 µl plasma was incubated for 30 minutes at 37˚C with 200 µl assay mix containing incubation buffer (0,025 M glycin/NaOH, pH 9.6), 4-p-nitrophenylphosphate and $MgCl_2$ at final concentrations of 1.25 and 2 mM respectively. The enzyme reaction was stopped by adding 1 ml 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 (Utrecht, The Netherlands).

TNFα quantitation

The porcine kidney cell line PK15 (CCL 33) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium with glutamax I (Invitrogen Corp, Breda, The Netherlands) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 10% fetal calf serum and 2% porcine serum (this is referred to as PK15 medium). $4x10^4$ PK15 cells were seeded per well in 96-well flat-bottom microtiter plates (Greiner Bio-one, Frickenhausen, Germany) in 100 µl PK15 medium. After 16 ± 2 h culture at 37°C in 5% CO_2, medium was removed and substituted with 50 µl of PK15 medium supplemented with 2 µg/ml actinomycin-D (Acros, Geel, Belgium). After 1 h incubation at 37°C, 5% CO_2, 50 µl of (diluted) plasma samples or recombinant porcine TNFα standards (R&D systems, Minneapolis, MN) (range 0.001 – 1 ng/ml) in PK15 medium were added to each well. The plates were incubated at 37°C, 5% CO_2 for an additional 16 ± 2 h. Cells were fixed for 10 min with 50 µl of 25% acetic acid,

75% methanol (VWR Int., Amsterdam, The Netherlands) and stained during 30 min with 50 µl 0.4% crystal violet (Acros, Geel, Belgium) in methanol. Plates were rinsed with distilled water, dried at 50°C for 4 h and optical densities were determined spectrophotometrically at 595 nm.

Data analysis

The plasma concentration curves of BIAP 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 ± SD. Statistical analysis was performed using chi-square test (for mice survival experiments) or two-sided unpaired students T-test (for pharmacokinetic and pharmacodynamic experiments in piglets) with data considered significant when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).

Results

Effects of BIAP treatment on mice injected with a lethal dose of bacteria.

To investigate the potentially therapeutic effects of BIAP *in vivo*, mice received a lethal dose of *E. coli* bacteria i.p. followed by either an i.v. injection of 1.5 unit BIAP (n=5) or saline (n=5). Figure 1 shows that 4 out of 5 animals that were given saline died, or body temperature dropped below 32˚C between 10 and 30 hours after inoculation of bacteria. In contrast, only 1 out of 5 animals receiving BIAP after inoculation of bacteria died between 36 and 48 hours. 48-Hours after bacterial challenge, body temperature of BIAP-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; $p<0.05$).

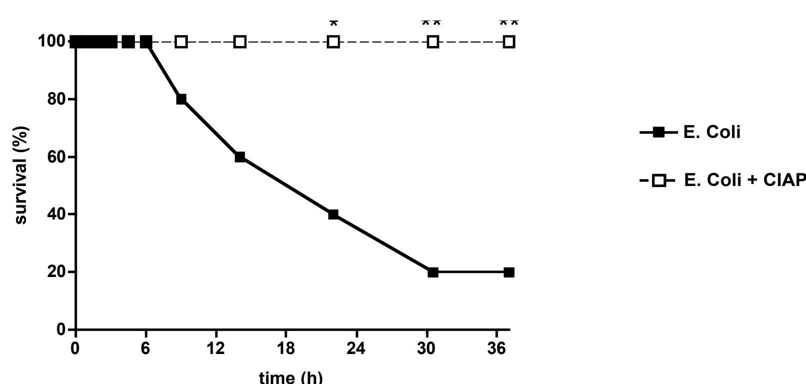

Figure 1: BIAP increases the survival of mice injected with a lethal dose of *E. coli* bacteria.

At t=0, 2.3×10^7 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 BIAP. Asterisks denote significant difference between the group injected with BIAP and the control group (*$p<0.05$, **$p<0.01$).

Basal AP levels in piglets

Before the pharmacokinetic experiments in piglets were started, basal plasma AP levels in two non-treated 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 BIAP, comparable to 10 or 100 times basal AP levels respectively.

Pharmacokinetic bolus experiments

In order to study the pharmacokinetic behavior of BIAP, piglets received a single i.v. bolus administration of 400 (n=5) or 4000 (n=6) units BIAP. At different time points after BIAP administration, plasma AP activity was determined. As shown in Figure 2, the plasma elimination curve of BIAP consists of 2 phases. Kinetic parameters were therefore calculated according to a two-compartment model and listed in Table I.

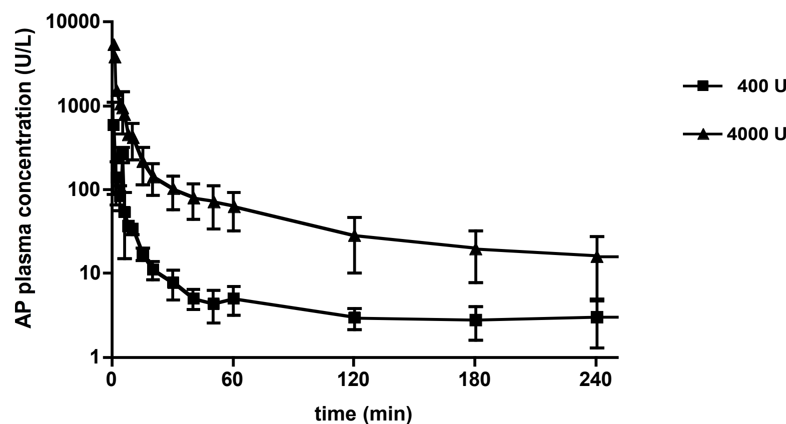

Figure 2: Plasma elimination curves of BIAP after a single i.v. bolus administration of 400 (n=5) or 4000 (n=6) units. At different time points after BIAP administration plasma AP activity was determined. Values depicted are the mean ± SD after correction for basal AP levels.

Table I: Pharmacokinetic parameters after a single i.v. bolus administration of 400 (n=5) or 4000 (n=6) units BIAP in piglets.

Dose (units)	$t_{1/2}$ 1 st [a]	$t_{1/2}$ 2 nd [b]	AUC (U x min/l) 1 st [a]	AUC (U x min/l) 2 nd [b]	Vd (l)	Cl [c] (l/min)
400	2’31” ± 0’50”	114 ± 82	2599 ± 2245	1213 ± 499	1.16 ± 1.22	0.27 ± 0.22
4000	3’52” ± 0’46” *	201 ± 102	7878 ± 3960 *	19922 ± 15548 *	1.90 ± 0.89	0.34 ± 0.14

[a] initial phase of elimination curve
[b] second phase of elimination curve
[c] initial clearance
* $p<0.05$ for 4000 units vs. 400 units

Pharmacokinetic infusion experiments

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

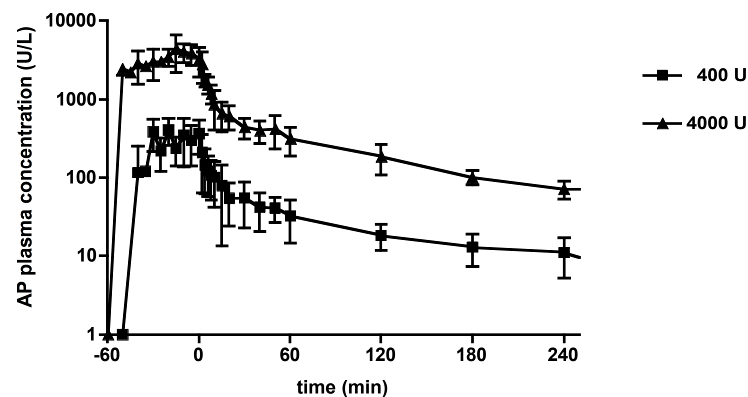

Figure 3: Plasma elimination curves of BIAP after steady state levels of 400 (n=5) or 4000 (n=4) units per liter. At different time points before and after BIAP administration, plasma AP activity was determined. Values depicted are the mean ± SD after correction for basal AP levels.

Table II: Pharmacokinetic parameters after a steady state of 400 (n=5) or 4000 (n=4) units BIAP per liter.

c[plasma] (units/l)	$t_{1/2}$ 1 st [a]	$t_{1/2}$ 2 nd [b]	AUC (u x min/l) 1 st [a]	AUC (u x min/l) 2 nd [b]	Vd (l)	Cl[c] (l/min)
400	4’51” ± 0’26”	142 ± 73	1852 ± 1121	7306 ± 3568	1.37 ± 0.53	0.19 ± 0.10
4000	5’16” ± 1’17”	192 ± 72	19781 ± 4991 ***	85581 ± 26202 ***	1.39 ± 0.50	0.18 ± 0.05

[a] initial phase of elimination curve
[b] second phase of elimination curve
[c] initial clearance
*** p<0.001 for 4000 units vs. 400 units

Multiple dosing experiments

Given the short $t_{1/2}$ of BIAP in plasma, it may be required for clinical application to apply repeated or continuous dosing regimes in LPS-overload conditions like sepsis. Therefore, 5 piglets received a single i.v. bolus administration of 4000 units BIAP every day for 28 consecutive days. Every day, blood samples were tested on plasma AP activity. Safety parameters were assessed twice a week. The results of the safety study are summarized in Table III. Administration of high doses of BIAP during 28 consecutive days had no statistical significant influence on hematological parameters, clinical chemistry and electrolyte balance.

Table III: Safety parameters after daily administration of 4000 units BIAP for 28 consecutive days (n=5).

Parameter		Days after initial injection					
		0	7	14	21	28	Reference values
Leucocytes [a]	(* 10^9/l)	17.6 ± 4.5	16.8 ± 2.7	18.6 ± 3.1	18.1 ± 3.7	18.8 ± 4.1	11 - 22
Lymphocytes [a]	(* 10^9/l)	7 ± 1	9 ± 2	8 ± 2	8 ± 2	9 ± 4	6 - 12.6
Thrombocytes [a]	(* 10^9/l)	370 ± 32	353 ± 86	341 ± 103	320 ± 76	340 ± 83	325 - 700
Ureum [b]	(mmol/l)	4.3 ± 0.9	3.8 ± 0.7	4.3 ± 0.8	4.1 ± 0.5	4.0 ± 0.3	3.0 - 8.5
Creatinin [b]	(μmol/l)	111 ± 13	114 ± 11	115 ± 14	109 ± 10	119 ± 14	90 - 240
γ GT [b]	(U/l)	23 ± 8	27 ± 4	28 ± 5	20 ± 6	25 ± 6	10 - 400
Sodium [c]	(mmol/l)	141 ± 5	144 ± 2	143 ± 2	142 ± 2	143 ± 3	140 - 150
Potassium [c]	(mmol/l)	4.4 ± 0.5	4.2 ± 0.3	4.6 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	4.7 - 7.1
Chloride [c]	(mmol/l)	101 ± 4	103 ± 2	102 ± 2	101 ± 1	100 ± 3	99 - 105
Calcium [c]	(mmol/l)	2.6 ± 0.1	2.7 ± 0.1	2.7 ± 0	2.6 ± 0	2.6 ± 0.2	1.8 - 2.9
Magnesium [c]	(mmol/l)	0.84 ± 0.07	0.85 ± 0.07	0.87 ± 0.05	0.85 ± 0.04	0.92 ± 0.04	0.8 - 1.6
Phosphate [c]	(mmol/l)	2.55 ± 0.30	2.62 ± 0.16	2.61 ± 0.15	2.69 ± 0.16	2.71 ± 0.21	1.6 - 3.4

[a] hematological parameters [b] clinical chemical parameters [c] elektrolytes

Reference values were obtained from the Animal Health Laboratory (AHL), University of Guelph, Canada http://ahl.uoguelph.ca/UsersGuide).

Effects of BIAP treatment on LPS-induced changes in clinical parameters

LPS-induced effects on clinical parameters showed to be dose-dependent and were back to normal 5-6 hours after LPS administration, whereas LPS+BIAP-treated animals showed a quicker recovery (data not shown).

Effects of BIAP treatment on LPS-induced hematological changes in piglets

As LPS administration is known to cause hematological changes in which high doses result in decreased leukocyte counts whereas low doses result in leukocytosis, the effect of LPS administration on blood cells was studied. Indeed, 10 µg LPS/kg reduced leukocyte counts whereas 50 ng LPS/kg caused an increase thereof (Table IV). Differential WBC counts showed a right-shift at two hrs (Figure 4A) and a left-shift at five hrs (Figure 4B) after 10 µg LPS/kg whereas at both time points left-shifts were observed after administration of 50 ng LPS/kg. Although BIAP had no effect on the decrease in leukocyte counts at 2 hrs after a high LPS dose, the left-shift five hrs after a high dose LPS was completely repressed. The effects of 50 ng LPS/kg were completely repressed by 4000 units BIAP (Figure 4, Table IV). The effects of 4000 units BIAP with 10 µg or 50 ng LPS/kg on differential blood cell counts are comparable to respectively 2 µg or 10 ng 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 BIAP (41 ± 4; $p<0.05$). Taken together, the above-mentioned results show that administration of BIAP resulted in up to 80% neutralization of the administered LPS.

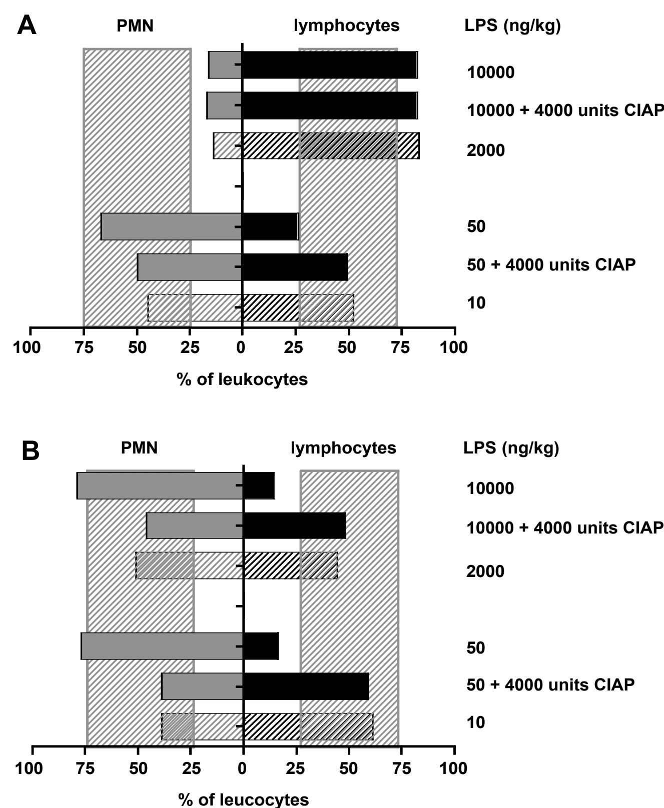

Figure 4: BIAP attenuates LPS-mediated effects on WBC counts. On t=0, two different LPS doses were administered with or without 4000 units BIAP. The percentage of PMN (gray) and lymphocytes (black) are shown at 2 (A) and 5 (B) hours after LPS administration. The shaded areas represent the 95% confidence interval of non-treated animals. Values outside these areas are considered significant ($p<0.05$).

Table IV: Relative leukocyte counts after treatment with LPS with or without BIAP

LPS dose (ng/kg)	Hours after injection	
	2 (%)	5 (%)
10000	19 ± 3	39 ± 19
10000 + 4000 units BIAP	20 ± 4	39 ± 27
50	157 ± 29	140 ± 16
50 + 4000 units BIAP	99	106

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 Figure 5, TNFα plasma levels were detected in all piglets injected with LPS with a dose-dependent maximum at approximately 1 hour after injection. 2-3 hours after LPS administration, TNFα levels were back to normal.

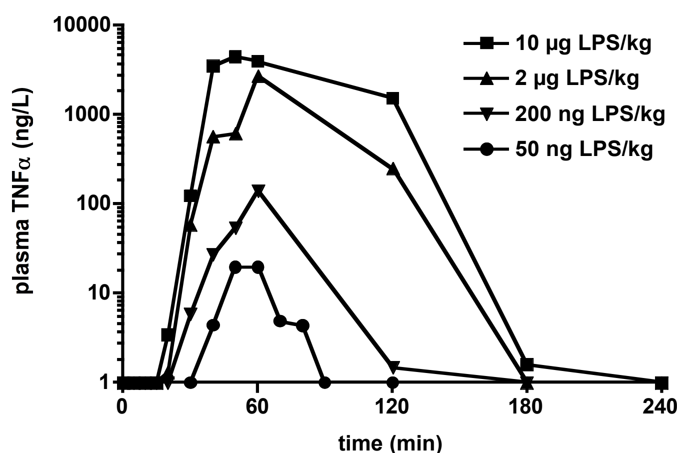

Figure 5: Plasma TNFα levels after a single i.v. bolus administration or infusion of different LPS doses (one piglet per dose). Doses of 10 and 2 µg LPS/kg bodyweight were administered as a single i.v. bolus whereas 200 and 50 ng LPS/kg bodyweight were administered as an i.v. infusion over 5 or 10 minutes, respectively. At different time points after LPS administration, plasma TNFα levels were determined. Values depicted are the results of 1 experiment.

Effects of BIAP treatment on LPS-induced TNFα release in piglets

Results show a clear difference in TNFα release in piglets treated with LPS alone compared to piglets treated both with LPS and BIAP (Figure 6). Comparison of TNFα activity over time (AUC) showed that infusion of 2500 units BIAP over 50 minutes reduces TNFα release after 10 µg LPS/kg bodyweight by 75% (Figure 6A). TNFα release after an infusion of 200 ng LPS/kg bodyweight is inhibited by 98% when administered during an infusion of 3000 units BIAP (Figure 6B). Simultaneous administration of 60 units BIAP and 50 ng LPS/kg bodyweight results in an inhibited TNFα release of 44% when compared to TNFα release after 50 ng LPS/kg bodyweight administration (Figure 6C).

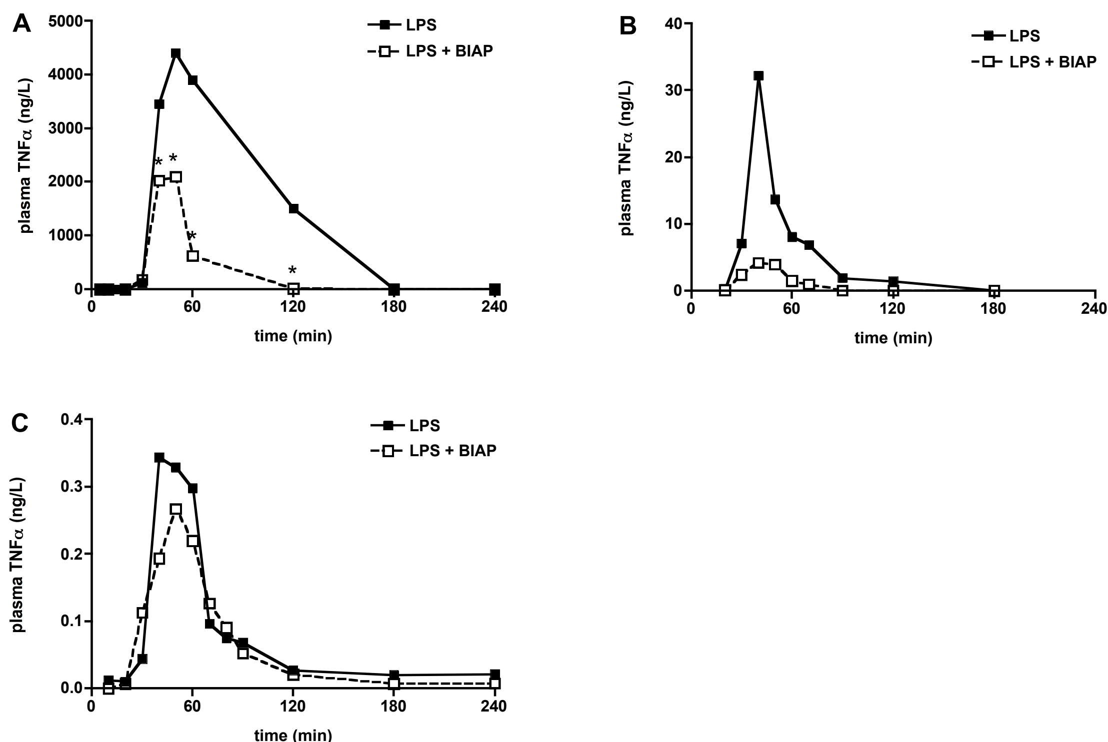

Figure 6: TNFα release after bolus administration of LPS with or without BIAP. A: At t=0, piglets were given a single i.v. bolus of 10 µg LPS/kg bodyweight (n=1) or an infusion of 2500 units BIAP over 50 minutes during which a bolus of 10 µg LPS/kg bodyweight was given at t=20 (n=1). B: At t=0, piglets were given an i.v. infusion of 200 ng LPS/kg bodyweight over 5 minutes (n=1) or an infusion of 3000 units BIAP over 50 minutes during which an infusion of 200 ng LPS/kg bodyweight was given from t=30-35 (n=3).
C: At t=0, piglets were given an i.v. infusion of 50 ng LPS/kg bodyweight over 10 minutes (n=1) or a single i.v. bolus administration of 20 units BIAP followed by an infusion of 40 units BIAP + 50 ng LPS/kg bodyweight over 10 minutes (n=2). At different time points TNFα plasma levels were determined. Asterisks denote significant difference between the 95% confidence interval of the group injected with BIAP and the control group (*$p<0.05$).

In Figure 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 BIAP can be deduced from Figure 7. This is done by projecting the TNFα response of LPS + BIAP on the LPS dose-response curve and by reading the corresponding LPS dose. The results show that BIAP can neutralize up to 80% of the administered LPS.

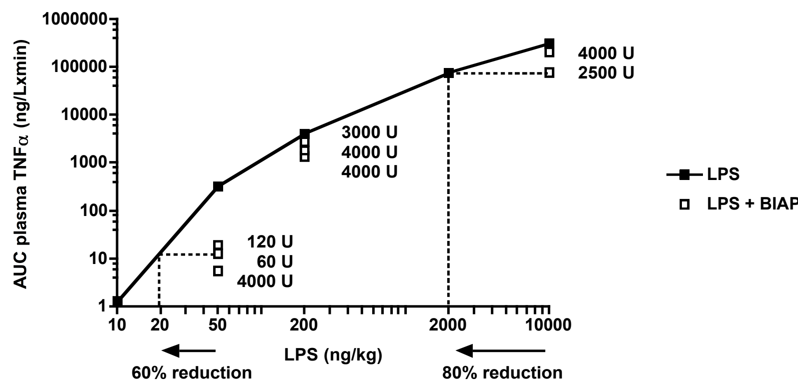

Figure 7: BIAP attenuates LPS-induced TNFα activity at different LPS doses. The figure depicts a summary of TNFα activity over time (AUC) from the experiments performed. LPS doses shown on the x-axis were administered with (open symbols) or without (closed symbols) BIAP in the concentration shown in the figure. Asterisks denote significant difference between the 95% confidence interval of the group injected with
BIAP and the control group (* $p<0.05$).

Discussion

Poelstra *et al.* [16,20] 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 HPLAP is able to detoxify lipopolysaccharide (LPS) by dephosphorylation of the lipid A moiety (W. Verweij, personal comm.). This results in its dephosphorylated counterpart monophosphoryl lipid A (MPLA), which was shown to be nontoxic and nonpyrogenic by Takayama *et al* [21,22]. 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.* [23] 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 BIAP as a therapeutic agent that attenuates LPS-mediated diseases. Towards this goal, mice were injected with a lethal concentration of *E. coli* bacteria alone or in combination with 1.5 units BIAP. 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 BIAP were determined.

Independent of the administered dose, a biphasic clearance with a fast initial and a slower second elimination of BIAP from the circulation was found. This is in agreement with results obtained by Scholtens *et al*. [24], who performed their experiments with canine intestinal AP in rats and Hoffmann *et al.* [25], who studied the kinetic behavior of feline and canine intestinal AP in cats and dogs, respectively.

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

Being a non-sialylated glycoprotein, BIAP is likely to be cleared by the asialoglycoprotein receptor (ASGPR) on liver cells, first discovered by Pricer and Ashwell [26,27]. Indeed, Scholtens *et al.* [24] and W. Verweij (personal comm.) 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 two hours. 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 re-entry of BIAP in the plasma compartment [24,28] . The first explanation, however, should show dose dependent plasma half life times, which we and others [24,25,29] did not observe.

Different from most other asialoglycoproteins, however, BIAP possesses a GPI anchor. It has been reported by Low *et al.* [30] and Medof *et al.* [31] that GPI anchored proteins bind to cell membranes. Due to the huge surface of the blood vessel endothelium, we hypothesize that administered BIAP binds to these endothelial cells in the first phase of clearance, resulting in a fast initial disappearance of BIAP 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 BIAP, 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 (see Table III). In addition, no accumulation of BIAP in the plasma was observed following the 28-day treatment (data not shown).

Having established the pharmacokinetic and safety profile of BIAP, the LPS detoxifying capacity of BIAP was studied in a piglet model. In this model, LPS was administered intravenously with or without BIAP. 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, BIAP was administered prior to LPS.

Administration of LPS to piglets induces hematological changes, which are mostly effects on WBC differentiation and thrombocytes [32,33]. Our results show that the dose-dependent effects of LPS on differential WBC counts and thrombocytes are inhibited by BIAP. LPS administration also resulted in a dose-dependent TNFα release in piglets, results that are supported by Norimatsu *et al.* [32] and Webel *et al.* [34]. BIAP administered prior to LPS inhibited TNFα release up to 98%.

In fact, two models were used, one for sepsis and one for inflammation, corresponding to high, respectively low LPS dose administration. In the sepsis model, 10 µg LPS/kg induced signs of severe sepsis, as specified in the APACHE score table [35]. These included tachycardia, fever, low WBC counts, low thrombocyte counts, etc. In contrast, 50 ng LPS/kg did not show signs of severe sepsis but signs of inflammation, such as fever and leukocytosis.

In both the sepsis and the inflammation model, BIAP attenuates LPS-toxicity. A striking observation in both models and for most parameters was that the effectiveness of LPS + BIAP was similar to that of 20% of that LPS dose, meaning that BIAP 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 LPS, the remaining toxicity has never been investigated.

Taken together the observed effects of BIAP administration on LPS-mediated changes in differential WBC counts, thrombocyte counts and TNFα release, the present study shows that BIAP neutralizes and thereby detoxifies LPS for up to 80%. While other anti-sepsis therapies aim at anti-inflammation or maintenance critical organ function, the function of BIAP 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 BIAP in LPS overload conditions like sepsis may add physiological aid in combating excess LPS before harmful effects may occur. In all experiments BIAP was administered prior to LPS exposure. This was done to prevent the immediate LPS toxicity through interaction with the respective LPS receptor [36]. We stress that alkaline phosphatase does not interact with thus ongoing LPS toxic cascade but rather prevents follow up toxicity of newly released LPS. Therefore it is proposed that BIAP 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 initiated.

References

1 Vincent JL, Carlet J, Opal SM. In: The sepsis text. Boston/Dorderecht/London: Kluwer Academic Publishers, 2002

2 Obana N, Takahashi S, Kinouchi Y, *et al.* Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, CD14. Scand J Gastroenterol 2002; 37:699-704

3 Rietschel ET, Wagner H. Pathology of septic shock: Springer-Verlag Berlin Heidelberg, 1996

4 de Haas CJ, van Leeuwen HJ, Verhoef J, *et al.* Analysis of lipopolysaccharide (LPS)-binding characteristics of serum components using gel filtration of FITC-labeled LPS. J Immunol Methods 2000; 242:79-89

5 Schumann RR. Recognition of bacterial endotoxin and modulation of the inflammatory response: the LBP/CD14 pathway during the acute phase. Sepsis 1998; 2:149-155

6 Triantafilou M, Triantafilou K. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol 2002; 23:301-304

7 Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001; 1:135-145

8 Barton GM, Medzhitov R. Control of adaptive immune responses by Toll-like receptors. curr opin immunol 2002; 14:380-383

9 Lin E, Lowry SF. The human response to endotoxin. Sepsis 1998; 2:255-262

10 Howe LM. Novel agents in the therapy of endotoxic shock. Expert Opin Investig Drugs 2000; 9:1363-1372

11 Dickneite G, Leithauser B. Influence of antithrombin III on coagulation and inflammation in porcine septic shock. Arterioscler Thromb Vasc Biol 1999; 19:1566-1572

12 Baumann H, Gauldie J. The acute phase response. Immunol Today 1994; 15:74-80

13 van der Poll T. Immunotherapy of sepsis. Lancet Infect Dis 2001; 1:165-174

14 Vesy CJ, Kitchens RL, Wolfbauer G, *et al.* Lipopolysaccharide-binding protein and phospholipid transfer protein release lipopolysaccharides from gram-negative bacterial membranes. Infect Immun 2000; 68:2410-2417

15 Bernard GR, Vincent JL, Laterre PF, *et al.* Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001; 344:699-709

16 Poelstra K, Bakker WW, Klok PA, *et al.* Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

17 Bentala H, Verweij WR, Huizinga-Van der Vlag A, *et al.* Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566

18 McComb RB, Bowers GN, Posen S. Alkaline phosphatases. New York: Plenum Press, 1979

19 Fishman WH. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

20 Poelstra K, Bakker WW, Klok PA, *et al.* A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Invest 1997; 76:319-327

21 Takayama K, Qureshi N, Raetz CR, *et al.* Influence of fine structure of lipid A on Limulus amebocyte lysate clotting and toxic activities. Infect Immun 1984; 45:350-355

22 Takayama K, Qureshi N, Ribi E, *et al.* Separation and characterization of toxic and nontoxic forms of lipid A. Rev Infect Dis 1984; 6:439-443

23 Koyama I, Matsunaga T, Harada T, *et al.* Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. Clin Biochem 2002; 35:455-461

24 Scholtens HB, Hardonk MJ, Meijer DK. A kinetic study of hepatic uptake of canine intestinal alkaline phosphatase in the rat. Liver 1982; 2:1-13

25 Hoffmann WE, Dorner JL. Disappearance rates of intravenously injected canine alkaline phosphatase isoenzymes. Am J Vet Res 1977; 38:1553-1556

26 Pricer WE, Jr., Ashwell G. The binding of desialylated glycoproteins by plasma membranes of rat liver. J Biol Chem 1971; 246:4825-4833

27 Pricer WE, Jr., Hudgin RL, Ashwell G, *et al.* A membrane receptor protein for asialoglycoproteins. Methods Enzymol 1974; 34:688-691

28 Kuhlenschmidt MS, Hoffmann WE, Rippy MK. Glucocorticoid hepatopathy: effect on receptor-mediated endocytosis of asialoglycoproteins. Biochem Med Metab Biol 1991; 46:152-168

29 Hoffmann WE, Renegar WE, Dorner JL. Serum half-life of intravenously injected intestinal and hepatic alkaline phosphatase isoenzymes in the cat. Am J Vet Res 1977; 38:1637-1639

30 Low MG, Zilversmit DB. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918

31 Medof ME, Nagarajan S, Tykocinski ML. Cell-surface engineering with GPI-anchored proteins. Faseb J 1996; 10:574-586

32 Norimatsu M, Ono T, Aoki A, *et al.* Lipopolysaccharide-induced apoptosis in swine lymphocytes in vivo. Infect Immun 1995; 63:1122-1126

33 Andonova M, Borissov I, Sotirov L. Changes in some factors of the innate immunity and serum zinc and iron concentrations in pigs following intravenous administration of Escherichia coli lipopolysaccharide. Onderstepoort J Vet Res 2001; 68:91-99

34 Webel DM, Finck BN, Baker DH, *et al.* Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J Anim Sci 1997; 75:1514-1520

35 Knaus WA, Draper EA, Wagner DP, *et al.* APACHE II: a severity of disease classification system. Crit Care Med 1985; 13:818-829

36 Ulevitch RJ. Recognition of bacterial endotoxins by receptor-dependent mechanisms. Adv Immunol 1993; 53:267-289

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In vitro detoxification of LPS by bovine calf intestinal alkaline phosphatase (BIAP), a novel therapeutic drug for LPS-mediated diseases

To be submitted

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Abstract

Several investigators have demonstrated that bovine intestinal alkaline phosphatase (BIAP) attenuates the lipopolysaccharide-mediated inflammatory response. Therefore, BIAP might represent a novel therapeutic agent in the treatment of LPS-mediated diseases like Gram-negative sepsis. Despite all the promising results obtained with BIAP *in vivo*, little is known about the mechanisms by which it exerts its biological activity. A bioassay in which the inflamed *in vivo* situation can be mimicked would be of great help in exploring BIAP mechanisms and the co-factors that are involved.

To test the LPS-detoxifying capacity of BIAP *in vitro*, macrophages and epithelial cells were incubated with LPS with or without BIAP, after which cell supernatants were screened on inflammatory parameters. In addition, assays were performed in which macrophages were stimulated with supernatant from epithelial cells incubated with LPS with or without BIAP.

The results of these studies clearly show that BIAP significantly reduces LPS-induced cytokine and NO_x production. It is suggested that, in order to exert its biological activity, BIAP needs to be associated to the cell membrane, most probably by means of its GPI-anchor.

Introduction

Lipopolysaccharide (LPS) is a molecule present in the outer cell membrane of Gram-negative bacteria and consists of a variable repeating oligosaccharide chain (O-antigen) and an oligosaccharide core, covalently bound to lipid A [1,2]. Lipid A contains two phosphate groups that are essential for the biological action of LPS [3,4]. Removal of a single phosphate group from lipid A results in the formation of monophosphoryl lipid A (MPLA), which is non-toxic and able to induce tolerance towards LPS due to its antagonistic properties [5-7].

Several acute and chronic diseases, for example Gram-negative sepsis, acute respiratory distress syndrome (ARDS) and ulcerative colitis (UC), are LPS-mediated [8-11] and characterized by the excessive production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNFα) and other inflammatory mediators (e.g. IL-8, NO_x) [12-14]. Many therapies against Gram-negative sepsis aimed at the neutralization or antagonization of pro-inflammatory cytokines or at the neutralization of LPS by using anti-LPS antibodies or LPS-binding proteins [15-19]. However, none of these trials have been proven effective.

Recently, several investigators have shown the promising therapeutic effects of alkaline phosphatase (AP) [20-23]. AP is an ectoenzyme attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, allowing enzymatic activity outside the cell. Soluble forms of AP, which are released from the cell membrane by phospholipases, are also found. [24] The mammalian alkaline phosphatase family consists of several isoforms of the enzyme, including the tissue-non-specific APs (liver-bone-kidney-type) and the tissue-specific APs (intestinal, placental, and germ-cell type) [25]. One of its physiological roles seems to be the removal of a single phosphate group from the lipid A moiety of LPS [26,27], thereby attenuating its toxicity.

Oral administration of LPS to rats resulted in a prolonged endotoxemia after inhibition of endogenous intestinal alkaline phosphatase [28]. In mice lethally infected with gram-negative bacteria, human placental alkaline phosphatase (HPLAP) and bovine calf intestinal alkaline phosphatase (BIAP) were able to reduce mortality [22, 27]. In piglets, BIAP treatment reduced LPS-induced TNFα levels [22].

So far, the mechanism by which AP detoxifies LPS is not fully understood. An *in vitro* assay would be helpful to investigate this mechanism and the possible necessary co-factors that facilitate this. Epithelial cells form the first line of defence in the body whereas macrophages that are attracted to sites were inflammation occurs serve a second line of defence. Therefore, these are cells of choice to study the effect of AP on LPS toxicity *in vitro*.

Whether BIAP is able to reduce LPS-induced cell responses *in vitro* is subject of this study. The effect of BIAP on LPS toxicity was tested in a direct activation assay using the murine macrophage cell-line RAW264.7 and the epithelial cell lines T84, A-549 and HEK293T. To represent the natural occuring response in which macrophages are attracted towards sites where inflammation occurs, indirect assays were performed. For this purpose macrophages were incubated with supernatant from epithelial cells stimulated with LPS with or without BIAP, after which the effect of BIAP on macrophage TNFα, IL-6 and NO_x production was determined.

Materials and methods

Test material

Bovine calf intestinal alkaline phosphatase (BIAP; GMP-processed, purity ALPIXG: 675 U/mg) was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of BIAP able to hydrolyse 1 μmole of p-nitrophenylphosphate per minute at pH 9.6 at 25˚C.

Reagents and media

RPMI-1640, DMEM (4.5 g/l glucose) and DMEM/F-12 (1:1) media (all containing glutamax I), penicillin, streptomycin, hepes, and sodiumpyruvate were from Invitrogen Corp. (Breda, The Netherlands). Foetal bovine serum (FBS) was obtained from Wisent Inc. (Quebec, Canada). *E. coli* LPS O111:B4 (prepared by phenol extraction and dissolved in sterile phosphate-buffered saline), sulphanylamide, n-naphtylethylenediamine, lactate dehydrogenase (LDH) assay, phosphatidylcholine, cholesterol and octylglucoside were from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). SM-2 Bio-Beads (20-50 mesh) were from BioRad (Veenendaal, The Netherlands) and extensively washed with methanol and water before use [29]. Actinomycin-D, crystal violet, and p-nitrophenylphosphate (pNPP) were from Acros Organics (Geel, Belgium). Human IL-8, murine IL-6 and TNFα ELISA kits were purchased from Biosource Europe SE (Nivelles, Belgium).

Cell culture

The murine macrophage cell line RAW264.7 and the human epithelial cell lines T84 (colorectal carcinoma) and HEK293T (embryonic kidney) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human epithelial cell line A-549 (lung carcinoma) and the human monocytic cell line THP-1 were a kind gift from drs. M.A. Dessing (AMC, Amsterdam). RAW264.7 and HEK293T cells were maintained in DMEM/F12 (1:1) medium. T84 cells were cultured in DMEM medium containing 4.5 g/l glucose, whereas A-549 and THP-1 cells were grown in RPMI-1640. All media contained Glutamax I and were supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Medium for RAW264.7 and HEK293T cells also contained 15 mM hepes and 1 mM sodiumpyruvate. Cells were grown in a humidified incubator at 37˚C and 5% CO_2. All epithelial cell lines were subcultured at confluence by employing trypsin-EDTA. RAW264.7 cells were scraped using a rubber policeman and THP-1 cells were diluted in fresh medium.

Direct activation of macrophages and epithelial cells

For activation assays, RAW264.7 were plated into 24-wells plates (Nunc, Roskilde, Denmark) at a concentration of $2x10^5$ cells/ml, and grown for 16 ± 2 hrs at 37°C and 5% CO_2. Medium was refreshed and cells were incubated with 1 μg LPS/ml for 24 hrs with or without 1 U BIAP/ml.

Epithelial cells were plated into 12-well plates (Nunc, Roskilde, Denmark) with 2 ml of medium. On reaching confluence, medium was refreshed and cells were incubated with 1 µg LPS/ml for 2 hrs (T84 and HEK293T) or 24 hrs (A-549) with or without 1 U BIAP/ml. In other experiments, cells were pre-incubated with 1 U BIAP/ml. After 2 (RAW and T84) or 24 (A-549) hrs, cells were rinsed and subsequently incubated with medium containing 1 µg LPS/ml for 24 hrs. Control cells were incubated with 1 µg LPS/ml alone. All cell supernatants were collected after 24 hrs and stored at –70˚C until further use.

Indirect activation of macrophages

RAW264.7 cells were cultured and plated as previously described. Medium was removed and 800 µl of fresh medium was added. 200 µl of supernatant from epithelial cells, which were previously incubated with 1 µg LPS/ml with or without 1 U BIAP/ml for 2 hrs (T84 and HEK293T) or 24 hrs (A-549), was transferred to RAW264.7 cells. After an incubation period of 24 hrs, all cell supernatants were collected and stored at –70˚C until further use.

BIAP incorporation in liposomes

Large unilamellar vesicles were prepared by freeze-thaw and extrusion procedure. Molar mixtures of PC/Chol (2:1) were solubilized at a concentration of 10 mg/ml in chloroform/methanol (2:1, v/v). The organic solvent was removed by a nitrogen flow under atmospheric pressure, after which the lipid film was resuspended in a 10 mM Tris-buffer, containing 150 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, pH 8.5 (TBS) at a concentration of 20 mg/ml. The hydrated lipid suspension was then exposed to six freeze-thaw cycles (-180˚C/+25˚C) and passed ten times through a 0.2 µm anopore filter (VWR International BV, Amsterdam, The Netherlands).

BIAP was reconstituted according to Levy *et al.* [30] and Angrand *et al.* [31]. PC/Chol liposomes (1 mg lipid/ml) were destabilized with 16 mM OG, followed by the addition of 100 U BIAP/ml (= 211 µg protein) and incubated for 30 minutes at 30˚C. OG was removed by hydrophobic adsorption on SM-2 Bio-Beads. For this purpose, 80 mg beads were added per ml, which were left to incubate for 2 hrs. The process was repeated twice, with incubations of 2 and 1 h respectively. In order to determine BIAP incorporation, proteoliposomes were submitted to centrifugation at 100.000 rpm for 1 h at 4˚C in a Beckman TLA 100.3 rotor. Supernatant and pellet were collected and analyzed for BIAP content by measuring its enzymatic activity.

Bioassay with liposomes

Aliquots of 10 µg LPS/ml were pre-incubated with BIAP, liposomes, BIAP and liposomes or BIAP incorporated in liposomes. The enzymatic BIAP activity used was 1 U/ml. LPS alone was used as a

control. After a 24-hour incubation period, aliquots were submitted to centrifugation at 100.000 rpm for 15 minutes at 4˚C in order to remove liposomes. RAW264.7 and THP-1 cells were incubated with 10-fold serial dilutions of the supernatant. After 1 and 2 hrs of incubation, 50 µl of cell supernatant was transferred to L929 cells for TNFα quantitation. After 24 hrs, RAW264.7 and THP-1 cell supernatants were collected and stored at –70˚C until further use.

TNFα quantitation

The murine fibroblast cell line L929 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. $4x10^4$ L929 cells were seeded per well in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in 100 µl medium. After 16 ± 2 hrs culture at 37°C and 5% CO_2, medium was removed and replaced by 50 µl of fresh medium supplemented with 2 µg/ml actinomycin-D. After 1 h incubation at 37°C and 5% CO_2, 50 µl of RAW264.7 or THP-1 cell supernatant was added to each well. The plates were incubated at 37°C, 5% CO_2 for an additional 16 ± 2 hrs. Cells were fixed for 10 min with 50 µl of 25% acetic acid, 75% methanol and stained during 60 min with 50 µl of 0.4% crystal violet in methanol. Plates were rinsed with distilled water and dried at 40°C, after which optical densities were determined spectrophotometrically at 595 nm.

Cytokine assays

Commercially available ELISA kits were used to determine concentrations of murine IL-6 and TNFα as well as human IL-8 in cell culture supernatants. Assays were performed according to the manufacturers' instructions.

Nitric oxide assay

Nitric oxide (NO_x) production was determined by means of the Griess method. In brief, 100 µl cell supernatant was incubated with 10 µl of a 10 mg/ml sulphanylamide solution in 5% phosphoric acid for 10 minutes. In addition, 10 µl of a 5.4 mM n-naphtylethylenediamine solution was added and incubated for another 3 minutes. Plates were read at 540 nm.

BIAP-binding assay

Epithelial cells were grown in 12-wells plates until confluence was reached. RAW264.7 cells were seeded at a concentration of 5×10^5 cells/ml in 12-well plates and grown for 16 ± 2 hrs. To study if BIAP was able to bind to the cells during activation experiments, medium was replaced and cells were incubated with 1 U of BIAP/ml for 2 hrs (T84, HEK and RAW) or 24 hrs (A-549). In addition,

cells were washed three times with PBS, after which the amount of BIAP bound to the cells was determined by measuring enzymatic activity.

Enzymatic detection of BIAP

BIAP activity was detected enzymatically by incubating the cells with 1 ml of an isotonic working solution containing 0.025 mM glycine, 8.62 mM $MgCl_2$ and 3.2 mM pNPP for 1 hr at 25 ˚C. The enzyme reaction was stopped by adding 500 µl of 2 M NaOH, after which the end product p-nitrophenol was quantitatively determined by measuring the extinction at 405 nm.

LDH release assay

In order to determine cell membrane permeability after incubation with isotonic working solution, LDH release was measured. For this purpose, aliquots of cell supernatant were taken after incubation with isotonic working solution. In addition cells were lysed, after which cell debris was pelleted by centrifugation. In aliquots from cell supernatant as well as in aliquots obtained after cell lysis, LDH content was determined according to the manufacturers' guidelines. Aliquots from lysed non-treated cells were included as a control for total LDH activity. Cell membrane permeability was determined as the ratio of LDH activity recovered in the supernatant and total cell LDH content.

Statistical analysis

Data are depicted as mean ± S.E.M. Statistical analysis was performed using two-sided unpaired students t-test with data considered significant when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).

Results

To determine the optimal LPS response, cells were incubated with different concentrations of LPS ranging from 1 ng/ml to 10 µg/ml. Figure 1 shows IL-6 production by RAW264.7 cells as a representative parameter. RAW264.7 cells respond to LPS by a dose-dependent IL-6 production. Stimulation of RAW264.7 cells with LPS concentrations of 1 and 10 µg LPS/ml resulted in a significantly enhanced IL-6 response whereas 100 ng/ml or lower only slightly increased IL-6 production. Therefore, all further *in vitro* assays were performed with an LPS concentration of 1 µg/ml.

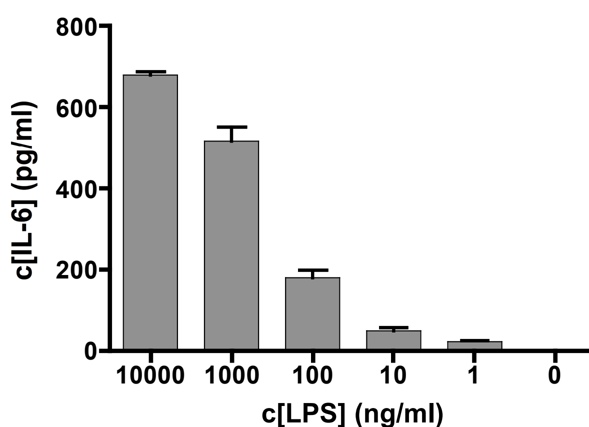

Figure 1:Dose-response curve of IL-6 production by RAW264.7 cells after exposure to LPS. Values are depicted as mean ± S.E.M. of 3 individual experiments.

Direct activation of macrophages and epithelial cells / direct bioassay

The test substance bovine calf intestinal alkaline phosphatase (BIAP) was examined for its capacity to reduce LPS-induced macrophage and epithelial cell responses *in vitro*. For this purpose, macrophages (RAW264.7) and epithelial cells (T84, A-549 and HEK293T) were incubated with 1 µg LPS/ml with or without 1 unit BIAP/ml, after which inflammatory parameters were determined. Incubation of RAW264.7 cells with 1 µg LPS/ml with or without 1 unit BIAP/ml for 24 hrs resulted in the production of the inflammatory parameters TNFα, IL-6 and NO_x, which is shown in Figure 2. Simultaneous incubation of LPS and BIAP did not result in decreased cytokine or NO_x levels. Pre-incubation of RAW264.7 cells with 1 unit BIAP for two hours, followed by washing and incubation of the cells with 1 µg LPS/ml had no effect on TNFα levels, whereas IL-6 and NO_x levels were reduced by 17% and 30%, respectively. After exposure to LPS for 24 hrs, A-549 cells and HEK293T cells produced IL-8 whereas T84 cells did not (see Figure 3). Incubation of A-549 cells with LPS and BIAP at the same time resulted in a 21% reduction in IL-8, whereas IL-8 produced by HEK293T cells incubated under the same conditions showed a 26% reduction.

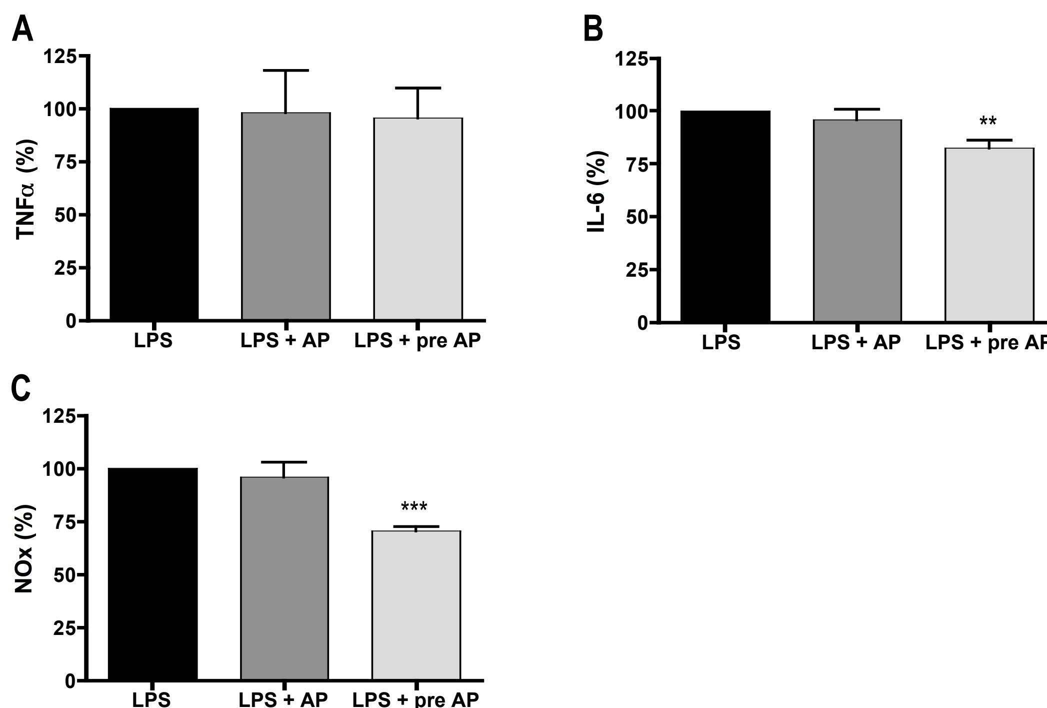

Figure 2: TNFa production (A), IL-6 production (B) and NO_x production (C) by RAW264.7 cells after exposure to 1 µg LPS/ml with or without 1 unit BIAP/ml. Cells treated with LPS only are set at 100%. Values are depicted as mean ± S.E.M. of 3 individual experiments. Asterisks denote significant difference between cells treated with LPS alone and cells treated with LPS + BIAP (* p<0.05, ** p<0.01, *** p<0.001).

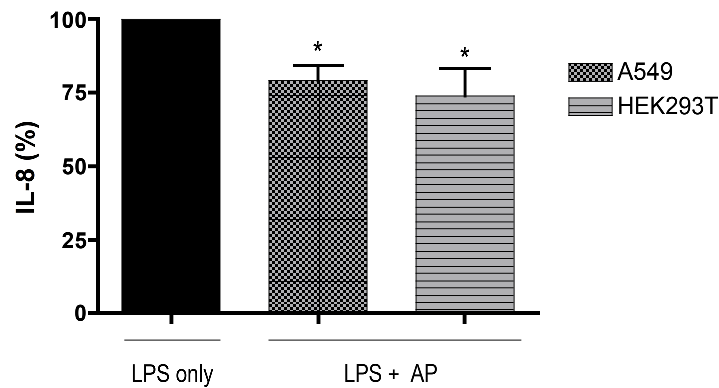

Figure 3: IL-8 production by A-549 and HEK293T cells after 24 hrs exposure to 1 μg LPS/ml with or without 1 unit BIAP/ml. Cells treated with LPS only are set at 100%. Values are depicted as mean ± S.E.M of 2 different experiments. Asterisks denote significant difference between cells treated with LPS alone and cells treated with LPS + BIAP (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Indirect activation of macrophages / indirect bioassay

To resemble the 'inflamed' *in vivo* situation more closely, a co-culture model with epithelial cells and macrophages was developed. In this model epithelial cells were incubated with LPS, followed by transfer of the supernatant to macrophages. After an additional 24 hrs incubation period on macrophages, inflammatory parameters were determined. Transfer of supernatants from epithelial cells stimulated with 1 μg LPS/ml to RAW264.7 cells resulted in the production of TNFα, IL-6 and NO_x. In contrast, transfer of supernatants from epithelial cells simultaneously incubated with LPS and 1 unit BIAP/ml to RAW264.7 cells resulted in a significantly reduced production of TNFα, IL-6 and NO_x, as is shown in Figures 4A-C. Incubation of T84, A-549 or HEK293T cells with LPS and BIAP resulted in a reduction of RAW264.7-produced TNFα by 44%, 29% and 17%, respectively (see Figure 4A). In addition, IL-6 production by RAW264.7 was reduced by 43%, 31% and 29% (see Figure 4B) whereas NO_x levels produced by RAW264.7 were reduced by 19%, 26% and 23% (see Figure 4C). Pre-incubation of epithelial cells with 1 unit BIAP/ml for two (T84 and HEK293T) or 24 (A-549) hours, followed by washing and incubation with 1 μg LPS/ml did not result in more reduced cytokine or NO_x levels when compared to cells incubated with LPS and BIAP simultaneously (data not shown).

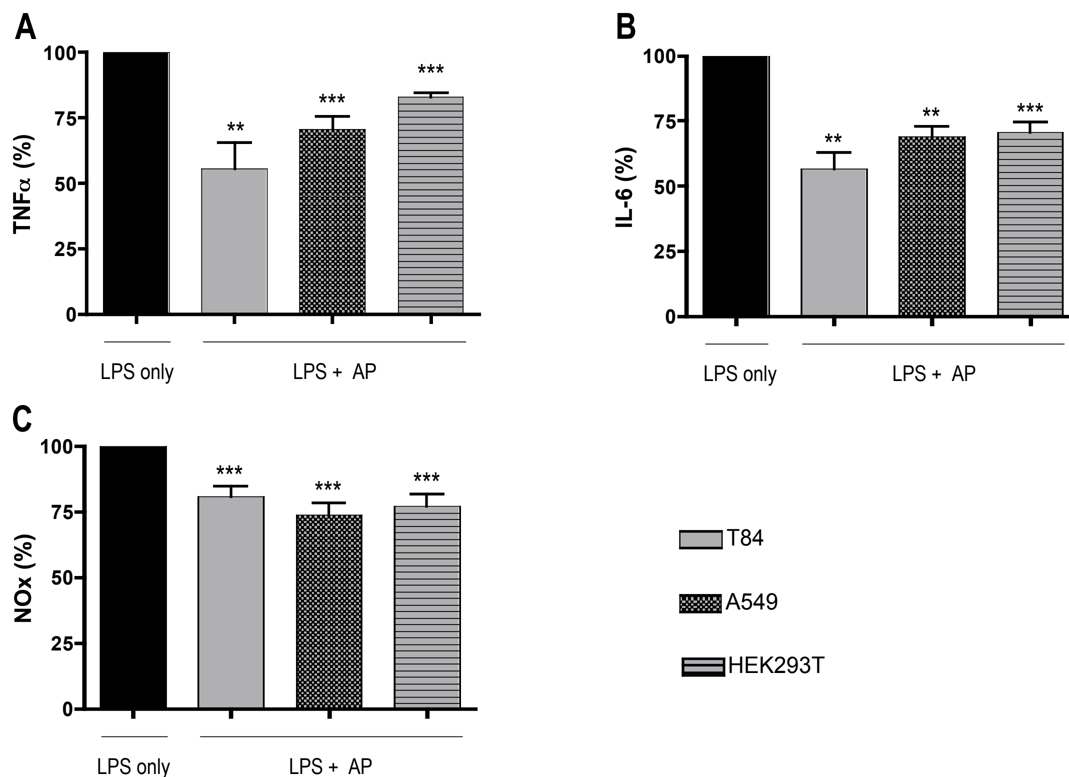

Figure 4: TNFα production (A), IL-6 production (B) and NO_x production (C) by RAW264.7 cells after supernatant transfer from T84, A-549 and HEK293T cells exposed to 1 μg LPS/ml with or without 1 unit BIAP/ml. Cells treated with LPS only are set at 100%. Values are depicted as mean ± S.E.M of at least 2 different experiments. Asterisks denote significant difference between cells treated with LPS alone and cells treated with LPS + BIAP (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Bioassay with liposomes

To investigate the hypothesis that BIAP might need a solid phase in order to exert its biological activity, BIAP was incorporated (= reconstituted) into the outer layer of liposomes. BIAP-containing liposomes were tested for their LPS-detoxifying capacity by incubating them with LPS in a Tris-buffer for 24 hrs, followed by transfer of the supernatant to RAW264.7 or THP-1 cells. After an additional 24 hrs incubation period on macrophages, inflammatory parameters were determined. Figure 5 shows that incubation of 1 μg LPS/ml with BIAP reconstituted into liposomes did not result in decreased TNFα, IL-6 and NO_x production. In addition, reconstituted BIAP was not able to reduce TNFα and IL-8 levels produced by THP-1 cells, as is shown in Figure 6.

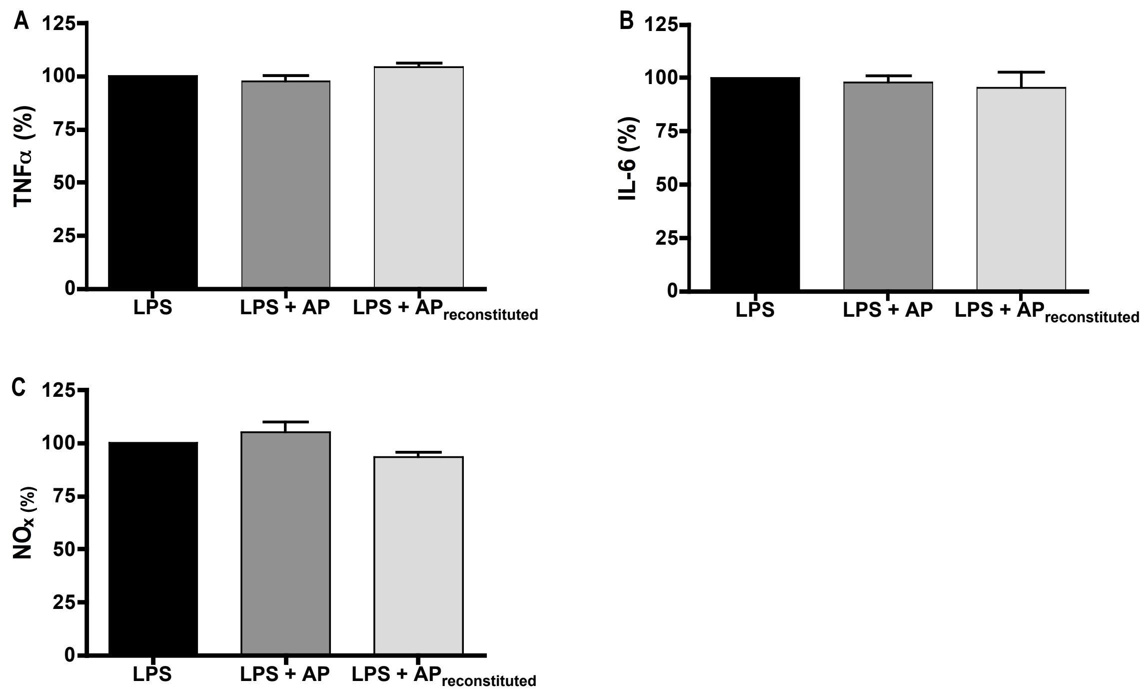

Figure 5: TNFα production (A), IL-6 production (B) and NO_x production (C) by RAW264.7 cells after exposure to 1 μg LPS/ml with or without 1 unit BIAP/ml. Cells treated with LPS only are set at 100%. Values are depicted as mean ± S.E.M. of at least 2 individual experiments

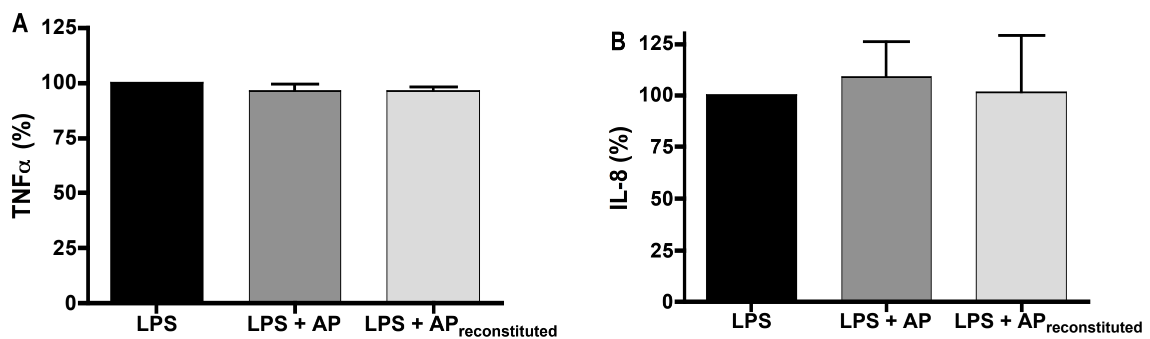

Figure 6: TNFα production (A) and IL-8 production (B) by THP-1 cells after exposure to 1 μg LPS/ml with or without 1 unit BIAP/ml. Cells treated with LPS only are set at 100%. Values are depicted as mean ± S.E.M. of 2 individual experiments.

BIAP-binding assay

To investigate the hypothesis that BIAP binds to the cells during the activation experiments, an assay was performed in which BIAP-binding was determined. For this purpose, T84, A-549, HEK293T and RAW264.7 cells were incubated with 1 U BIAP/ml. After thorough rinsing, cell-associated BIAP was visualized enzymatically. Figure 7 shows that enzymatic activity on T84, A-549 and RAW264.7 cells incubated with BIAP was approximately 2 times higher than that on cells not incubated with BIAP. For HEK293T cells incubated with BIAP, this increase was about 3.5 times. To examine if the measured BIAP activity was outside the cells, an LDH release assay was performed. The results from this assay show that incubation of cells with an isotonic working solution results in marginal, and therefore negligible, increases in membrane permeability (data not shown). The results from the LDH assay combined with the observation that pNPP does not penetrate the cell membrane [32] leads us to suggests that the measured BIAP activity is located outside the cell.

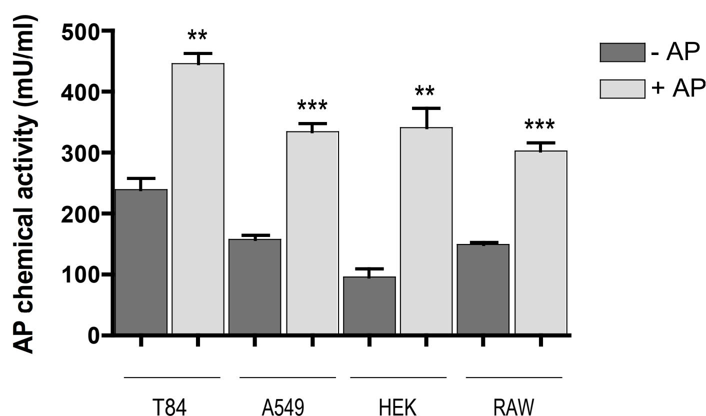

Figure 7: Binding of BIAP to T84, A-549, HEK293T and RAW264.7 cells.

Shown is the increase in BIAP enzymatic activity of cells incubated with 1 unit BIAP/ml when compared to cells not incubated with BIAP. Values are depicted as mean ± S.E.M. of 3 individual experiments. Asterisks denote significant difference between cells incubated with BIAP and cells incubated without BIAP (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Discussion

Alkaline phosphatase is capable of removing a single phosphate group from LPS, which results in its less toxic counterpart monophosphoryl LPS [26]. Recently, several investigators have described the LPS-detoxifying effect of AP *in vivo* [21-23,28,33]. Besides the *in vivo* studies with AP, Koyama *et al.* [28] also performed studies with rat intestinal AP *in vitro.* They showed that after dephosphorylation of LPS by IAP, endothelial cell viability was four times higher when compared to cells incubated with non-dephosphorylated LPS.

So far, the mechanism by which AP detoxifies LPS is not fully understood. The aim of the present study therefore was to develop an *in vitro* assay in which this mechanism could be further

explored. Epithelial cells are present at sites where Gram-negative bacteria may enter the body (e.g. lung and gastro-intestinal tract). Macrophages are a part of the second line of defence in the body. Both cell types produce all kinds of inflammatory mediators upon LPS stimulation, and were therefore used to study the LPS-detoxifying capacity of BIAP *in vitro*.

In the first part of the study, the effect of BIAP on LPS-induced cell responses was tested in a direct activation assay using the murine macrophage cell-line RAW264.7 and the three human epithelial cell lines T84 (colon), A-549 (alveolar) and HEK293T (kidney). Simultaneous incubation of 1 µg LPS/ml and 1 U BIAP/ml on macrophages for 24 hours did not have any reducing effect on cytokine or NO_x levels whereas LPS stimulation of epithelial cells in the presence of BIAP resulted in significantly reduced IL-8 levels. An explanation for this might be the presence or absence of proteins involved in the LPS signaling cascade, like TLR4. Since integrity of the epithelial surface of the body must be warranted at all times, it is important not to have any unwanted inflammatory responses ongoing. It is therefore that TLR4 is absent or down regulated on epithelial cells. Expression of this molecule is up regulated when large amounts of bacteria or their products are present at the epithelial linings, which results in the initiation of an immune response. Epithelial cells can also internalize LPS after which it can bind to intracellular soluble TLR4 and activate the cell. When bacteria or their products cross the epithelial linings and reach the blood, macrophages have to respond immediately in order to prevent the body from further harm. Since macrophages do express TLR4 on their surface, the presence of LPS results in direct activation, which might make it more difficult for AP to interact with, and detoxify LPS.

In the second part of the study macrophages and epithelial cells were pre-incubated with BIAP, followed by the removal of BIAP and subsequent exposure to LPS. For macrophages this resulted in decreased IL-6 and NO_x levels whereas TNFα levels were not affected. A possible explanation for this may be that the TNFα, IL-6 and NO_x inducing capacities of LPS and dephosphorylated LPS are different. Aybay and Imir [10] showed that for LPS and MPLA the TNFα inducing capacities were similar whereas IL-6 and NO_x inducing capacities of MPLA were lower than those for LPS. Since AP dephosphorylates the lipid A part of LPS, these findings may also explain our results. In contrast to the observed additional effect of BIAP pre-incubation on LPS-induced macrophage responses, this strategy did not result in an additional detoxification effect on epithelial cells. The presence/absence of the molecules involved in LPS-signaling might also explain these differences. When LPS is present near the RAW264.7 cell membrane with BIAP bound to it, competition can occur between TLR4 and BIAP, resulting in a reduced production of inflammatory mediators. LPS present near epithelial cells with BIAP bound to it will be dephosphorylated by BIAP and not bind to TLR4 since this molecule is almost not expressed on epithelial cells.

An explanation for the striking difference in detoxifying capacity observed between simultaneous incubation of macrophages with BIAP and LPS and pre-incubation of macrophages with BIAP, followed by washing and subsequent incubation with LPS might be found in the structure of the protein. BIAP is a GPI-anchored protein and the presence of such a protein in an

aqueous solution is energetically not favourable. Low and Zilversmit [34] and Medof *et al.* [35] reported that GPI anchored proteins bind to cell membranes. Based on this we hypothesize that a) when BIAP is present in, for example, cell culture medium, it immediately binds to the cell membrane and b) that this binding is essential for BIAP to exert its full biological activity. To test this hypothesis, experiments were performed in which macrophages and epithelial cells were incubated with BIAP after which cell-bound BIAP was visualized enzymatically. The results show that after BIAP incubation, BIAP activity associated with the cells is increased when compared to cells not incubated with BIAP. Experiments by DePierre and Karnovski [32] showed that the substrate used to enzymatically visualize AP, pNPP, does not penetrate the cell membrane. This, together with the observation that incubation with an isotonic substrate solution does not result in increased membrane permeability, leads us to conclude that the BIAP activity measured in this study was associated with the outside of the cell membrane. However, by using this experimental setup it cannot be excluded that cells internalize BIAP during incubation.

To further explore the hypothesis that BIAP must be attached to a solid phase in order to exert its biological activity, BIAP was incorporated in the outer membrane of liposomes (=reconstituted). However, in liposomes reconstituted BIAP was not able to reduce levels of inflammatory parameters produced by RAW264.7 or THP-1 cells. This result, combined with the finding that BIAP reduces LPS-induced responses on RAW264.7 and epithelial cells and the fact that BIAP immediately incorporates into cell membranes, suggests that BIAP does need a solid phase to exert its biological activity. This solid phase is most probably a cell membrane where possible necessary co-factors are present.

The last part of this study describes the effect of indirect stimulation of macrophages. In an inflamed *in vivo* situation, epithelial cells produce several kinds of proteins that serve as chemoattractants, like IL-8, MCP-1 and MIP-1. These chemoattractants recruite macrophages and PMNs to the infection site, resulting in the production of other inflammatory mediators. To more closely reflect this situation, indirect assays were performed in which the supernatant from epithelial cells stimulated with LPS with or without BIAP was transferred to macrophages. The thus observed significant decreases in TNFα, IL-6 and NO_x levels produced by the macrophages were interpreted as the net result of LPS-BIAP interaction on epithelial cells.

In conclusion, this study clearly shows that BIAP attenuates LPS-mediated cell responses in different *in vitro* test systems. Previously, several investigators have shown the very promising effects of AP on LPS toxicity *in vivo*. Anti-sepsis therapies using antibodies in order to neutralize LPS result in abrogation of the inflammatory response, which is not preferable when the causative agent (bacterium) is still present. In contrast, dephosphorylation of LPS by AP results in the formation of its non- or less toxic counterpart monophosphoryl LPS (MP-LPS). The thus formed MP-LPS acts as a partial antagonist by modulating the inflammatory response. BIAP might therefore represent a novel therapeutic in the treatment of LPS-mediated diseases.

References

1 Holst O, Thomas-Oates J, Brade H. Preparation and structural analysis of oligosaccharide monophosphates obtained from the lipopolysaccharide of recombinant strains of Salmonella minnesota and Escherichia coli expressing the genus-specific epitope of Chlamydia lipopolysaccharide. Eur J Biochem 1994; 222:183-194

2 Rietschel ET, Kirikae T, Schade FU, et al. Bacterial endotoxin: molecular relationships of structure to activity and function. Faseb J 1994; 8:217-225

3 Qureshi ST, Gros P, Malo D. The Lps locus: genetic regulation of host responses to bacterial lipopolysaccharide. Inflamm Res 1999; 48:613-620

4 Schromm AB, Brandenburg K, Loppnow H, et al. Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion. Eur J Biochem 2000; 267:2008-2013

5 Astiz M, Rackow E, Still J, et al. Pretreatment of normal humans with monophosphoryl lipid A induces tolerance to endotoxin: a prospective, double-blind, randomized, controlled trial. Crit Care Med 1995; 23:9-17

6 Yao Z, Foster PA, Gross GJ. Monophosphoryl lipid A protects against endotoxic shock via inhibiting neutrophil infiltration and preventing disseminated intravascular coagulation. Circ Shock 1994; 43:107-114

7 Wy CA, Goto M, Young RI, et al. Prophylactic treatment of endotoxic shock with monophosphoryl lipid A in newborn rats. Biol Neonate 2000; 77:191-195

8 Luster M, Germolec D, Yoshida T, et al. Endotoxin-induced cytokine gene expression and excretion in the liver. Hepatology 1994; 19:480-488

9 Grewe M, Gausling R, Gyufko K, et al. Regulation of the mRNA expression for tumor necrosis factor-alpha in rat liver macrophages. J Hepatol 1994; 20:811-818

10 Aybay C, Imir T. Comparison of the effects of Salmonella minnesota Re595 lipopolysaccharide, lipid A and monophosphoryl lipid A on nitric oxide, TNF-[alpha], and IL-6 induction from RAW 264.7 macrophages. FEMS Immunol and Med Microbiol 1998; 22:263-273

11 Ou J, Carlos TM, Watkins SC, et al. Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. Nitric Oxide 1997; 1:404-416

12 Vincent J, Carlet J, Opal S. The Sepsis Text. Dordrecht: Kluwer Academic Publishers, 2002

13 Obana N, Takahashi S, Kinouchi Y, et al. Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, CD14. Scand J Gastroenterol 2002; 37:699-704

14 Goodman RB, Pugin J, Lee JS, et al. Cytokine-mediated inflammation in acute lung injury. Cytokine Growth Factor Rev 2003; 14:523-535

15 Riedemann NC, Ward PA. Anti-inflammatory strategies for the treatment of sepsis. Expert Opin Biol Ther 2003; 3:339-350

16 Reinhart K, Karzai W. Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned. Crit Care Med 2001; 29:S121-125

17 Pollack M, Ohl CA, Golenbock DT, et al. Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induced proinflammatory functions. J Immunol 1997; 159:3519-3530

18 von der Mohlen M, Kimmings A, Wedel N, et al. Inhibition of endotoxin-induced cytokine release and neutrophil activation in humans by use of recombinant bactericidal/permeability-increasing protein. J Infect Dis 1995; 172:144-151

19 Lynn WA. Anti-endotoxin therapeutic options for the treatment of sepsis. J Antimicrob Chemother 1998; 41 Suppl A:71-80

20 Poelstra K, Bakker WW, Klok PA, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

21 Verweij WR, Bentala H, Huizinga-van der Vlag A, et al. Protection against an Escherichia coli-induced sepsis by alkaline phosphatase in mice. Shock 2004; 22:174-179

22 Beumer C, Wulferink M, Raaben W, et al. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. J Pharmacol Exp Ther 2003; 307:737-744

23 van Veen SQ, van Vliet AK, Wulferink M, et al. Bovine intestinal alkaline phosphatase attenuates the inflammatory response in secondary peritonitis in mice. Infect Immun 2005; 73:4309-4314

24 McComb R, Bowers G, Posen S. Alkaline Phosphatases. New York: Plenum Press, 1979

25 Fishman W. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

26 Poelstra K, Bakker W, Klok P, et al. A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Investig 1997; 76:319-327

27 Bentala H, Verweij WR, der Vlag AH-V, et al. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566

28 Koyama I, Matsunaga T, Harada T, et al. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. Clin Biochem 2002; 35:455-461

29 Holloway PW. A simple procedure for removal of Triton X-100 from protein samples. Anal Biochem 1973; 53:304-308

30 Levy D, Gulik A, Bluzat A, et al. Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. Biochim et Biophys Acta 1992; 1107:283-298

31 Angrand M, Briolay A, Ronzon F, et al. Detergent-mediated reconstitution of a glycosyl-phosphatidylinositol-protein into liposomes. Eur J Biochem 1997; 250:168-176

32 DePierre JW, Karnovsky ML. Ecto-enzymes of the Guinea Pig polymorphonuclear leukocyte. J Biol Chem 1974; 249:7121-7129

33 Poelstra K, Bakker W, Klok P, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

34 Low M, Zilversmit D. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918

35 Medof M, Nagarajan S, Tykocinski M. Cell-surface engineering with GPI-anchored proteins. FASEB J. 1996; 10:574-586

4

Species differences in safety of bovine calf intestinal alkaline phosphatase (BIAP), a potential novel therapeutic drug for attenuating lipopolysaccharide (LPS)-mediated diseases

In preparation

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Abstract

Bovine calf intestinal alkaline phosphatase (BIAP) has previously been demonstrated to attenuate the lipopolysaccharide (LPS)-mediated inflammatory response in mice and piglets. Therefore, BIAP might represent a novel therapeutic agent in the treatment of LPS-mediated diseases like Gram-negative sepsis.

Before BIAP can be applied in the treatment of Gram-negative sepsis, safety and tolerability of its administration had to be investigated. For this purpose, single-dose and multiple-dose safety studies were performed in mice and dogs. Since these animals showed such diverse reactions on the protein, more species were included in the study.

Single dose administration of BIAP did not result in any adverse effects in mice, rats, dogs, piglets and humans. Multiple-dose BIAP administration resulted in antibody formation and serum sickness in mice and guinea pigs but not in piglets, dogs and humans. This study therefore suggests that subjects that consume or have consumed bovine products in their food can be therapeutically treated with BIAP.

In addition to studying the safety of BIAP administration in various species, pharmacokinetics of BIAP in man were studied. BIAP clearance was shown to be dose-independent and showed a biphasic pattern with an initial fast distribution phase and a second, much slower, elimination phase with plasma half-life times ranging from 1.68 to 7.9 hours.

Introduction

Alkaline phosphatases, dimeric membrane-bound ectoenzymes common to all organisms, can be found in many tissues throughout the body. In mammals this group of enzymes can be classified as either tissue-specific, which include placental (PLAP), germ cell (GCAP) and intestinal (IAP) isoenzymes, or tissue non-specific (TNAP) which includes the bone-liver-kidney (BLK) isotype [1]. Alkaline phosphatases catalyze the hydrolysis of phosphomonoesters with release of inorganic phosphate at alkaline pH *in vitro* [2] and have been ascribed several biological functions. For instance, experiments with TNAP knockout mice implied a role for TNAP in bone mineralization and vitamin B6 utilization [3]. Also, inactivation of the gene would result in infantile hypophosphatasemia and perinatal death. Beckman [4-7] suggested a role for the PLAP isoenzyme in maternal-fetal interactions. It was also shown that PLAP can act as F_c fragment receptor, thereby suggesting a role for capture of circulating IgG [8]. For IAP, Flock and Bollman [9] were the first to report about increased levels after lipid feeding. Much later, Mahmood *et al.* [10] suggested a role for IAP in transepithelial lipid transport.

In 1997, Poelstra *et al.* [11] showed that human placental alkaline phosphatase (HPLAP) dephosphorylates and thereby detoxifies LPS at physiological pH levels. LPS is a constituent of the outer membrane of Gram-negative bacteria and essential for bacterial growth and survival [12]. Many Gram-negative bacterial species routinely inhabit the normal human gastrointestinal tract. However, enhanced colonization of the gastrointestinal tract by these Gram-negative bacteria by direct invasion and/or translocation of bacteria (or LPS) across the gastrointestinal mucosa can lead to Gram-negative sepsis. The presence of LPS and its ability to interact with host immune cells accounts for much of the clinical threat associated with Gram-negative sepsis. Gram-negative sepsis is characterized by the excessive production of proinflammatory cytokines like TNFα, IL-1, IL-6, IL-8 and IL-12 [13,14], activation of proteolytic cascades, coagulation abnormalities [15] and hemodynamic responses, resulting in hypotension, poor tissue perfusion and, finally, multi organ failure [16,17].

Despite the exploration of many anti-sepsis therapies in the past, most of the successes obtained in animal models could not be reproduced in patient trials. Recently, the anticoagulant and anti-inflammatory agent activated protein C (APC) was reported to reduce overall mortality in severe sepsis patients with 6 % [18].

Poelstra *et al.* and Bentala *et al* showed that HPLAP reduces mortality in mice lethally infected with Gram-negative bacteria, thereby suggesting a role for alkaline phosphatase in protection against Gram-negative sepsis [19, 20]. Recently, we showed that also bovine calf intestinal alkaline phosphatase (BIAP) detoxifies LPS [21]. In the latter study, mice survived a lethal *E. coli* infection after BIAP administration whereas in piglets hematological changes and TNFα responses were antagonized after doses up to 10 μg LPS per kg body weight. Therefore it was proposed that BIAP could represent a novel therapeutic drug in the treatment of Gram-negative sepsis and other LPS-mediated diseases [21].

Before BIAP can be applied in the treatment of Gram-negative sepsis, safety and tolerability of its administration had to be investigated. For this purpose, single-dose and multiple-dose safety studies were performed in mice and dogs. Since these animals showed such diverse reactions on the protein, more species were included in the study. Also, a study on tolerability as well as the pharmacokinetic behavior of BIAP in man was performed.

Materials and methods

Test material

Bovine calf intestinal alkaline phosphatase (BIAP; GMP-processed, purity ALPIXG: 675 U per mg protein, Biozyme, Blaenavon, UK)

Chemicals

4-p-nitrophenylphosphate was obtained from Acros (Geel, Belgium). Streptavidin pe-cytochrome 5 was obtained from DAKO chemicals (Glostrup, Denmark). Rat IgG2a + IgG2b antibodies conjugated with either FITC, PE or RPE-Cy5, FITC conjugated rat anti-mouse CD3, PE conjugated rat anti-mouse CD16, biotin conjugated rat anti-mouse CD19, PE conjugated rat anti-mouse CD4, biotin conjugated rat anti-mouse CD8 and purified rat anti-mouse IgE (clone R35-72), were obtained from Pharmingen (San Diego, USA). Purified goat anti mouse IgG1, purified anti-human Ig(H+L) and anti-human IgE were from Southern Biotech Ass Inc. (Birmingham, AL, USA).

Animal study design

A summary of the design of all animal studies is given in Table I (page 81). All studies were performed at TNO Nutrition and Food Research (Zeist, The Netherlands) according to Organisation for Economic Cooperation and Development (OECD) Principles for Good Laboratory Practice (as revised in 1997), Paris, ENV/MC/CHEM (98)17.

Animals

Specific pathogen-free animals: male and female CD-1 mice (5-6 weeks old), male and female Wistar rats (4-5 weeks old) and male Dunkin Hartley guinea pigs (3 weeks old) were obtained from Charles River Deutschland, Sulzfeld, Germany whereas 6-8 weeks old male Dunkin Hartley guinea pigs were from David Hall, UK. Male and female beagle dogs were from Harlan, The Netherlands (5 months old) or from Harlan-Winkelmann, Germany (11-18 months old), respectively. All animals except the dogs were randomly assigned to specific treatment groups. Dogs were allocated proportionally to bodyweight in such a way that allocation of more than one dog from one litter to the same test group was avoided. In one case, however, two female dogs from the same litter were assigned to the same dose group. All animals were housed under

conventional conditions (males and females separately) with *ad libitum* access to drinking water and allowed to acclimatize for 7-14 days. Rodents were kept in macrolon cages with sterilized wood shavings as bedding material with five animals per cage (female mice and rats), 5-6 per cage (guinea pigs) or one per cage (male mice; to avoid fighting) at the animal housing facility of TNO Nutrition and Food Research (Zeist, The Netherlands) with food *ad libitum*. Dogs were housed in pens (connected stainless steel cages with concrete floors) at the animal housing facility of Utrecht University (GDL), two or four dogs of the same group and sex per pen. They received portions of 300 g of food in the morning. Female Dutch Landrace x Yorkshire piglets were obtained from Proefaccommodatie de Tolakker, Utrecht University (Utrecht, The Netherlands) at 8 weeks of weaning and were individually housed under conventional conditions at the animal housing facility of Utrecht University (GDL). Once a day they received a commercial pig diet served as sludge whereas drinking water was provided *ad libitum* (for details: see [21]).

Methods

Determination of AP Activity   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 plasma was incubated for 30 min at 37°C with 200 µl assay mix containing incubation buffer (0.025 M glycine/NaOH, pH 9.6), 4-p-nitrophenylphosphate, and $MgCl_2$ 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.

Analysis of BIAP specific IgE and IgG1 antibodies

BIAP-specific antibodies were measured in serum obtained at day 14 (dogs) or day 28 (mice, guinea pigs and piglets). Costar high-binding ELISA plates were coated overnight at 4 ± 2°C with 100 µl carbonate buffer containing either 2 µg/ml purified rat anti-mouse IgE or 2 µg/ml purified goat anti mouse IgG1. Next day, and after blocking with 150 µl TBST/BSA for 2 hours, plates were washed with TBS/0.05% Tween (TBST) and samples (diluted in TBST/BSA; first dilution = 2^3) were added and incubated for 1 hr at 37±2°C. After washing, plates were incubated with 100 µl TBST/BSA containing 10 µg/ml BIAP. Finally, ELISA plates were washed 3 times with TBST and 1 time with diethanolamine. 100 µl alkaline phosphatase substrate [pNPP, 1 mg/ml in diethanolamine buffer (9.7 % diethanolamine (v/v) and 0.02 % $MgCl_2$ (w/v) in distilled water, pH 9.8)] was added per well. After 30 minutes, the reaction was stopped using 50 µl of a 10% (w/v) EDTA solution and the plates were read at 405 nm on a microplate reader. Samples with an OD smaller than the OD measured for the first sample dilution (2^3) were below the detection limit. Samples with an OD bigger than the OD measured for the blanc sample + 3xSD were designated "positive".

(Histo)pathological analysis in mice All animals were subjected to macroscopic examination for pathological changes. A thorough necropsy was also performed on animals that died intercurrently (if not precluded by cannibalism or autolysis) or that had to be killed because they were moribund. As soon as possible after dissection, adrenals, brain, heart, kidneys, pituitary, prostate, seminal vesicles (with coagulation glands), spleen, liver, lungs, ovaries, testes, thymus, thyroids (with parathyroids) and uterus were weighed (pared organs together). Samples of the following tissues and organs were preserved in a neutral aqueous 4% phosphate-buffered formaldehyde dilution: adrenals, aorta, brain (brain stem, cerebrum, cerebellum), caecum, colon, epididymidis, eyes (with optic nerve), heart, joint/knee (femur), kidneys, larynx, lungs, liver, lymph nodes (cervical and mesenteric), mammary gland, nerve-peripheral (sciatic), ovaries, oviducts, oesophagus, pancreas, pituitary, prostate, rectum, salivary glands (sublingual, submaxillary, parotid), seminal vesicles + coagulation glands, skin, small intestine (duodenum, ileum, jejunum), spleen, spinal cord (at three levels), stomach (glandular and non-glandular), sternum + bone marrow, skeletal muscle (thigh), testes, thymus, thyroids with parathyroids, trachea/bronchi, tongue, urinary bladder, uterus, vagina and all gross lesions. The tissues to be examined microscopically were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Histopathological examination by light microscopy was performed on all preserved organs of all animals that were sacrificed on day 11 (interim necropsy), all animals of the control group and low-dose group that were sacrificed on day 28 (final necropsy), and of all animals that died during the study or were killed in extremis. In addition, histopathological examination was performed on relevant gross lesions of animals of all groups. PAS staining was performed on a few selected kidneys.

Immunophenotyping in mice Single cell suspensions were prepared from spleens removed at final necropsy. After washing, white blood cell numbers were determined using an automated hematology analyzer (K-800, Sysmex Toa, Kobe, Japan). Viability was determined by Acridine Orange/Ethidium Bromide labeling. Using fluorescently labeled monoclonal antibodies against CD3 (T cells), CD4 (T helper cells), CD8 (T suppressor/cytotoxic cells), CD16 (NK cells) and CD19 (B cells), white blood cells were phenotyped by flow cytometric analysis (Beckman Coulter Epics XL-MCL).

Hematology and clinical chemistry in mice and dogs Blood samples for hematology were collected in tubes containing EDTA and screened for hemoglobin, packed cell volume, prothrombin time, red blood cells, reticulocytes, thrombocytes and white blood cells (all by ABX Pentra 120 hematology analyzer). White blood cells were characterized by microscopic examination of blood smears stained according to Pappenheim. For clinical chemistry, blood samples were collected in heparinized tubes, after which plasma was prepared by centrifugation. ALP, ALAT, ASAT, GGT, glucose, total protein, albumin, urea, creatinin, bilirubin total, total cholesterol, triglycerides, phospholipids, calcium, sodium, potassium, chloride and inorganic phosphate were determined by using a Hitachi-911 analyzer with Roche reagent.

Preclinical single-dose

Mice (5/sex/group) BIAP was diluted in sterile 0.9% saline and administered in the tail vein as a slow bolus (duration up to 2 minutes) at a dose level of 5354 U/kg body weight. Animals were necropsied after a 14-day treatment-free period. Clinical observations, growth and gross examination at necropsy were used as criteria for disclosing possible harmful effects.

Rats (5/sex/group) BIAP was diluted in sterile 0.9% saline and administered in the tail vein by intravenous infusion at a dose level of 2677 U/kg body weight. Animals were necropsied after a 14-day treatment-free period. Clinical observations, growth and gross examination at necropsy were used as criteria for disclosing possible harmful effects.

Dogs (4 dogs total). Animals were anaesthetized (induction) with 15 ml propofol (Rapinovet®, intravenously). After this, dogs were intubated and anesthesia was maintained by inhalation anesthesia with isoflurane (Forene®, 2-3%) in combination with an O_2 (30%) / N_2O (70%) mixture. Stability of anesthesia was monitored and fluid support was provided with Lactated Ringers Solution (Braun Melsungen AG, Germany) via an infusion line in the vena cephalica (5-10 ml/kg bw/hr). Body temperature was monitored continuously by a Yellow Springs Y 400 temperature probe. After application of catheter, flow probe and ECG pad, the dog was turned over on its right side and covered with a blanket to prevent temperature loss. All catheters were continuously flushed with 0.9% NaCl + 2500 IE heparin (2-4 ml/hr) to keep them open. All substances were administered via the infusion line in the vena cephalica. Treatment started with the administration of the vehicle control, followed by the test substance at 15, 75 and 750 U/kg body weight. Finally, the positive control substance (xylazine, 2 mg/kg body weight) was administered. Cardiovascular parameters (Cardiac output, heart rate, ECG and systolic, diastolic and mean blood pressure) were measured and recorded over 45 minutes after administration. Immediately after the last measurement dogs were euthanized by an intravenous barbiturate overdose.

Preclinical multiple-dose

Mice (5/sex/group) BIAP was diluted in sterile 0.9% saline and administered in the tail vein at 75, 750 and 7500 U/ kg body weight once daily for 28 consecutive days. In order to perform hematology (day 24) or clinical chemistry (day 28), blood samples were taken by orbital punction whilst under CO_2/O_2-anaesthesia. Hematology and clinical chemistry were not performed when orbital punction was considered too aggravating for the animals. Necropsy of all remaining animals of all four experimental groups was performed on day 28 after a 4-hour period of fasting. Mice were killed by exsanguination from the orbital plexus while under CO_2/O_2-anaesthesia.

Guinea pigs BIAP was diluted in sterile 0.9% saline and administered intraperitoneally or intravenously in the penile vein at day 0, 2, 4, 7, 9 and 11 for sensitization and once intravenously at day 28 for challenge experiments. Doses used for sensitization were 5 and 50 µg BIAP protein

(3,38 and 33,8 U/animal; all n=6), challenge was performed with 5 mg BIAP protein (3380 U/animal). As a positive control, OVA was used in a dose equal to the highest BIAP dose (n=4).
ASA test The animals subjected to ip and iv sensitization treatment were intravenously challenged and observed continuously during a 30-minutes period after injection and thereafter for a further three hours for the following anaphylactic signs: piloerection, nose licking or scratching, unrest, tremor, sneezing or coughing, respiratory difficulties, ataxia, convulsions and death.
PCA test Naïve recipient animals were given intra dermal (id) injections of 0.1 ml undiluted serum derived from sensitized animals. Each test serum was given to two different animals, 10 id injections at most per animal. Id injections were followed by the iv challenge dose four hours later. Challenge was with 1 ml/kg body weight of a 1:1 mixture of BIAP or OVA (5 mg/ml) in 0.9% saline and a solution of 2% Evans blue dye. The cutaneous reactions were observed within 30 minutes after challenge. When staining was considered maximal, the diameter and the intensity of the blue spots at the injection sites were measured. The response was considered positive when the diameter of the blue area was larger than 5 mm and graded from weak (+) to moderate (++) and intense (+++).

Piglets For a detailed description of the safety study performed in piglets, see [21]. In brief, the jugular vein of each piglet was catheterized in order to facilitate the administration of test substance and blood sampling in piglets. After a recovery period, BIAP was diluted in sterile 0.9% saline and administered as an intravenous bolus of 200 U/kg body weight once daily for 28 consecutive days (n=5). Although piglets did not show any signs of toxicity after treatment with BIAP, stored blood samples taken on day 28 were used in the present study for testing for specific anti-BIAP antibodies.

Dogs Animals received 15 or 75 U BIAP/kg body weight once daily for 14 consecutive days in the cephalic/saphenous vein. Besides the tests enlisted in Table I (page 81), eye examination by ophthalmoscopic observations as well as electrocardiogram recordings were made towards the end of the treatment period. Necropsy was performed at day 14.

Statistical analysis

The statistical procedures used in the evaluation of the data were as follows:
Body weight: one-way analysis of covariance (covariate: body weight on day 0) followed by Dunnett's multiple comparison tests. Red blood cell and clotting potential variables, total white blood cell counts, clinical chemistry values, volume and density of the urine, immunophenotyping variables and organ weights: one-way analysis of variance (Anova) followed by Dunnett's multiple comparison test (mice) or Least Significant Difference test (dogs). Independent from the results of Anova, the homogeneity of variance was tested by means of Bartlett's test (dogs). If the

variance differed significantly ($p<0.01$), the variables involved were re-evaluated with Kruskal-Wallis non-parametric Anova followed by Mann-Whitney U-tests. Reticulocytes, relative differential white blood cell counts, urinary parameters except for volume and density: Kruskal-Wallis non-parametric Anova followed by Mann-Whitney U-tests. Histopathological changes: Fisher's exact probability test. All tests were two-sided. Probability values of $p<0.05$ were considered statistically significant.

Clinical study design

A summary of the design of all human studies is given in Table I (page 81). All studies were performed by SGS (Antwerp, Belgium) according to the principles of the "Declaration of Helsinki", the EMEA/CPMP position statement on the use of placebo in clinical trials with regard to the revised "Declaration of Helsinki" and in accordance with the Guideline for Good Clinical Practice. Study protocols were approved by the Medical Ethics committee – O.C.M.W., Antwerp.

Subjects

38 healthy volunteers were selected on the following criteria: Male, Caucasian, aged 18 to 50 years old, body mass index between 18 and 28 kg/m^2, no history of clinically significant allergies, no co-medication, not smoking more than 10 cigarettes eq per day, and liver enzyme levels within normal range. During the study none of the subjects withdrew.

Methods

Randomization

Recruited subjects were given a number between 1 and 38, chronologically. Subjects 1 to 8 were assigned to group 1, 9 to 16 to group 2, 17 to 24 to group 3, 25 to 32 to group 4, and 33 to 38 to group 5. Within each group, each subject was assigned to either BIAP or placebo according to computer generated randomization codes (SAS software, Procedure "Plan").

Treatment

The study was designed as a dose escalation study with five groups of six to eight subjects, receiving the active drug in a 10 min continuous iv infusion (7.5, 22.5 or 67.5 units BIAP/kg body weight/10 min), a continuous 24 hours iv infusion (200 units BIAP/kg body weight/24 hr) or a 10 min infusion at 67.5 U/kg body weight followed by 72 hr at 177.5 U/kg body weight/day (= 600 units BIAP/kg body weight/72 hr). The medication was prepared by the hospital pharmacist in syringes labelled with the subject code in order to blind subjects and medical staff (double blinded). Each subject was infused with a dose corresponding to its group and body mass, beginning during the morning.

Specific toxicity assessment

Before, during and after medication, blood samples were drawn and the patient was observed and asked in a non-suggestive manner (e.g. How do you feel?) for adverse effects. Blood samples were analyzed for alkaline phosphatase activity (pharmacokinetics), hematology, clinical chemistry and urinalysis. Vital signs (blood pressure and heart rate) were also recorded for all subjects. Electrocardiographs were taken at screening, prior to BIAP/placebo infusion, at different time points after the start of the infusion, at the end of the infusion and at the day 14 follow up visit. On day 0, day 14 and day 90, serum samples were analyzed for anti-BIAP antibodies (IgE and total Ig). Also, a skin prick test was performed on day 90.

Pharmacokinetics

At different time points, blood for BIAP screening was taken from the arm opposite to which BIAP was infused and collected in heparin tubes. Samples were centrifuged and stored at 4-8˚C until further analysis. AP determination was performed as described in "Animal study design" under methods: determination of AP activity.

Hematology and clinical chemistry

Blood for hematology was collected in EDTA vacutainers. Samples were analysed on hemoglobin, hematocrit, red blood cells, total white blood cells, leucocyte differential count and platelets by the Clinical and Haematology Laboratory of Hospital Stuivenberg (Antwerp). For clinical chemistry, blood was collected in SST® Gel and Clot Activator tubes (BD). Samples were screened on sodium, potassium, calcium, inorganic phosphate, total protein, albumin, glucose, total cholesterol, triglycerides, blood urea nitrogen (BUN), creatinin, uric acid, total bilirubin, alkaline phosphatase, ASAT, ALAT, gamma GT and LDH at the Central Clinical Laboratory (CKVL) of the Hospital Stuivenberg (Antwerp).

Assessment of immunogenicity

At the screening visit and post-study visits (day 14 and 90), blood was collected in heparinised tubes. Samples were analysed for the presence of anti-BIAP specific immunoglobulins. In brief, Costar high binding ELISA plates were coated overnight at 4 ± 2°C with 50 µl PBS containing either 2 µg/ml purified anti-human Ig or 2 µg/ml purified anti-human IgE in PBS. Next day and after blocking with 250 µl blocking buffer per well for 2 hours, (diluted) samples were added and incubated for 1 hour. Plates were washed, 50 µl blocking buffer containing antigen (BIAP, 10 µg/ml) was added per well and plates were incubated for an additional hour. Finally, plates were washed 4 times with PBS/0.05% tween and once with 100 µl diethanolamine buffer (9.7 % diethanolamine (v/v) and 0.02 % $MgCl_2$ (w/v) in distilled water, pH 9.8). 100 µl alkaline phosphatase substrate (1 mg pNPP/ml diethanolamine buffer) was added to each well and the reaction was stopped with 50 µl 50 mM EDTA (in distilled water). Plates were read at 405 nm on a microplate reader.

At 90 days after drug administration, a skin prick test was performed using a BIAP solution of 100 U/ml. As positive and negative control, a 1 mg/ml histamine hydrochloride solution or a 0.9% NaCl solution, respectively, were included. The size of the wheal and flare were measured after 15 minutes. A reaction of 3 mm greater than the negative control was regarded as positive.
Grading was as follows: + (no wheal, 3 mm flare), ++ (2-3 mm wheal with flare), +++ (3-5 mm wheal with flare) and ++++ (>5 mm wheal, may have pseudopodia).

Statistical analysis
Safety and tolerability in man were evaluated using descriptive statistics. Incidences of all adverse events were reported using medical dictionary for regulatory activities (MedDRA) coding (www.meddramsso.com) per treatment group. Pharmacokinetics of BIAP were evaluated according to a two-compartment model.

Results

Single-dose safety
The test substance bovine calf intestinal alkaline phosphatase (BIAP) was examined for possible acute intravenous toxicity in mice, rats and dogs. For this purpose, animals intravenously received different doses of test substance or vehicle.
Mice   Single dose administration of up to 5453 U BIAP per kg body weight did not result in clinical abnormalities, changes in body weight or well being, or in abnormalities at necropsy in any of the mice of the treatment and control groups (data not shown).
Rats   Administration of single BIAP doses of up to 2677 U per kg body weight did not induce any changes in body weight or well being, or in abnormalities at necropsy in any of the rats of the treatment and control groups (data not shown).
Dogs   The effect of intravenous administration of single BIAP doses on cardiovascular parameters was studied in anaesthetized dogs. Administration of up to 750 U BIAP per kg body weight did not result in changes in any of the cardiovascular parameters measured (data not shown).

Multiple dose safety
To study possible sub-acute toxic effects of BIAP, mice and dogs were once daily intravenously dosed with test substance or vehicle for 14 (dogs) or 28 (mice) consecutive days. All animals were observed daily after dosing and, if necessary, handled to detect signs of toxicity.

Mice   As shown in Table II (page 82), multiple intravenous administrations of BIAP to mice resulted in clinical abnormalities. In the mid-dose (750 U/kg body weight) and high-dose

(7500 U/kg body weight) group, two male and two female and four male and seven female animals respectively, died at day eight of the study whereas all surviving animals showed severe signs of toxicity (sluggishness, blepharospasm and ruffled skin). After the next BIAP administration (day 9), more animals died. Since mortality occurred within one hour after treatment, animals in the mid- and high-dose groups were not treated any further, whereas treatment of control and low-dose animals was continued. Also, an extra necropsy was included at day 11, which involved three male and three female mice of all treatment groups. After this (interim) necropsy, no females were left in the high-dose group.

Interim necropsy (day 11) This was performed on animals killed at day 11 and animals that had died earlier. Animals scheduled for this necropsy were given BIAP shortly before killing. Blood was collected for clinical chemistry and animals were subjected to macroscopic examination for pathological changes. Hematology, urinalysis and immunophenotyping were not performed on these animals. As shown in Table III (page 83), mid-dose females had statistically significant decreases in total protein, albumin, albumin to globulin ratio and phospholipids and statistically significant increases in urea, creatinin and triglycerides. Similar changes were observed in the one remaining mid-dose male. Chloride levels were significantly increased in low-dose females and decreased in mid-dose females. Table IV (page 84) shows significantly increased relative liver weights in mid- and high-dose females. A dose-related increase in relative kidney weights was observed in females, reaching significance in the high-dose group. In one low-dose, four mid-dose and ten high-dose animals, pathological macroscopic examination showed tail lesions consisting of encrustations, inflammation and, in severe cases, necrosis and loss of the tail distal to the injection site. Animals that died intercurrently had pathological changes in several organs and tissues. In the majority, the lungs exhibited increased cellularity consisting of mononuclear cells, including alveolar macrophages, polymorphonuclear cells and erythrocytes. In a few animals pulmonary haemorrhages were present and two animals showed (peri)vasculitis of the lung arteries. All these pulmonary changes point towards pulmonary inflammation. Microscopic examination of the tail lesions showed perivascular hemorrhages, and/or inflammation, focal necrosis or (necrotising) inflammation of all soft tissues. Kidneys of high-dose animals showed a significantly increased incidence of hypertrophy of distal tubular epithelial cells of the renal cortex. This abnormality was also found in a few mid-dose animals. To reveal possible glomerular basement membrane thickening, a few selected kidneys from mid- and high-dose animals were stained with PAS, which revealed no further abnormalities.

Final necropsy (day 28) When comparing low-, mid- and high-dose animals with controls, it should be noted that control and low-dose group animals were treated for the entire 28 days whereas the mid- and high-dose group animals were not treated during the last 20 days of the study. Since proper controls are absent, these groups were excluded from the final study results.
After multiple dose BIAP administration, no statistically significant differences were observed in hematological and clinical chemistry values, urine sample values and immunophenotyping of

spleen cells (data not shown). Table IV (page 84) shows no differences in relative body- and organ weights between control and low-dose groups on day 28. Five low-dose males and four low-dose females showed tail lesions consisting of encrustations, inflammatory aspect and, in severe cases, necrosis and loss of the tail distal to the injection site. Microscopic examination of control and low-dose animals killed at day 28 did not reveal renal changes. Sera of control and low-dose animals were also (qualitatively) tested on the presence of anti-BIAP antibodies and found positive only in BIAP-treated animals (titer > 2^8). In the sera from control animals no anti-BIAP antibodies were detected (detection limit = 2^3).

Guinea pigs
The ability of BIAP to induce the formation of reaginic antibodies after ip and iv sensitization treatment was investigated in guinea pigs using the active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) tests. All animals of the iv high dose group showed clinical signs associated with anaphylactic reactions, like lethargy, convulsions and dyspnoea, which remained for up to 30 minutes after injection of BIAP. Since two animals of this dose-group died after injection of BIAP, treatment was discontinued. In the ASA test on day 28, BIAP challenge resulted in severe signs of anaphylaxis in both the ip and iv sensitized animals. After death of two animals in each group, remaining animals were not challenged for animal welfare reasons. Sera from BIAP-sensitized animals contained reaginic antibodies as was evident from the positive responses in the PCA tests. Negative control (saline) did not give rise to a positive response in the ASA and PCA tests.

Piglets Previous experiments with BIAP revealed no toxicity after a 28-day treatment period [21]. Sera obtained on day 28 of this study were stored at –80°C and retrospectively tested on anti-BIAP antibodies and found negative (titer < 2^3).

Dogs Administration of up to 75 units of BIAP/kg body weight once daily for 14 consecutive days did not result in any changes in clinical well-being and cardiovascular parameters (data not shown), or hematology and clinical chemistry (Table V, page 85). Sera were also tested on anti-BIAP antibodies and found negative (titer < 2^3).

Clinical safety and pharmacokinetics in man
Adverse effects, as indicated by subjects receiving either placebo or BIAP or observed by the investigators, were evaluated by an independent medical doctor and all scored unrelated or unlikely to be related to BIAP (data not shown). Hematology (Table VI, page 86), clinical chemistry (Table VII, pages 87-89), and physical examination, 12-lead electro-cardiogram, urinalysis and vital signs (data not shown) showed no abnormalities. No anti-BIAP antibodies (specific Ig and IgE) were found in day 14 and day 90 post-treatment sera. Skin prick tests at the end of the study were also found negative.

To determine pharmacokinetic parameters of BIAP in man, healthy volunteers (n=5-6 per dose) received a bolus injection, administered as a 10 min infusion, or a continuous 24 or 72 h infusion containing different BIAP doses. Plasma elimination of BIAP after a bolus injection as well as after a 24 or 72 h continuous infusion was shown to be biphasic (Figures 1a, b, and c) and dose independent (Table VIII, page 89). Plasma half-life times showed high individual variation ranging from 1.68 to 7.90 h. Two subjects of the 10 min infusion group who had plasma half-life times of 33.5 and 29.4 h, respectively, were shown to have hepatic problems (Gilbert's disease [22]) and were therefore retrospectively excluded from the study. The mean volume of distribution was dose independent and the group averages ranged from 0.418 to 1.15 l/kg, implicating distribution of BIAP over a second compartment.

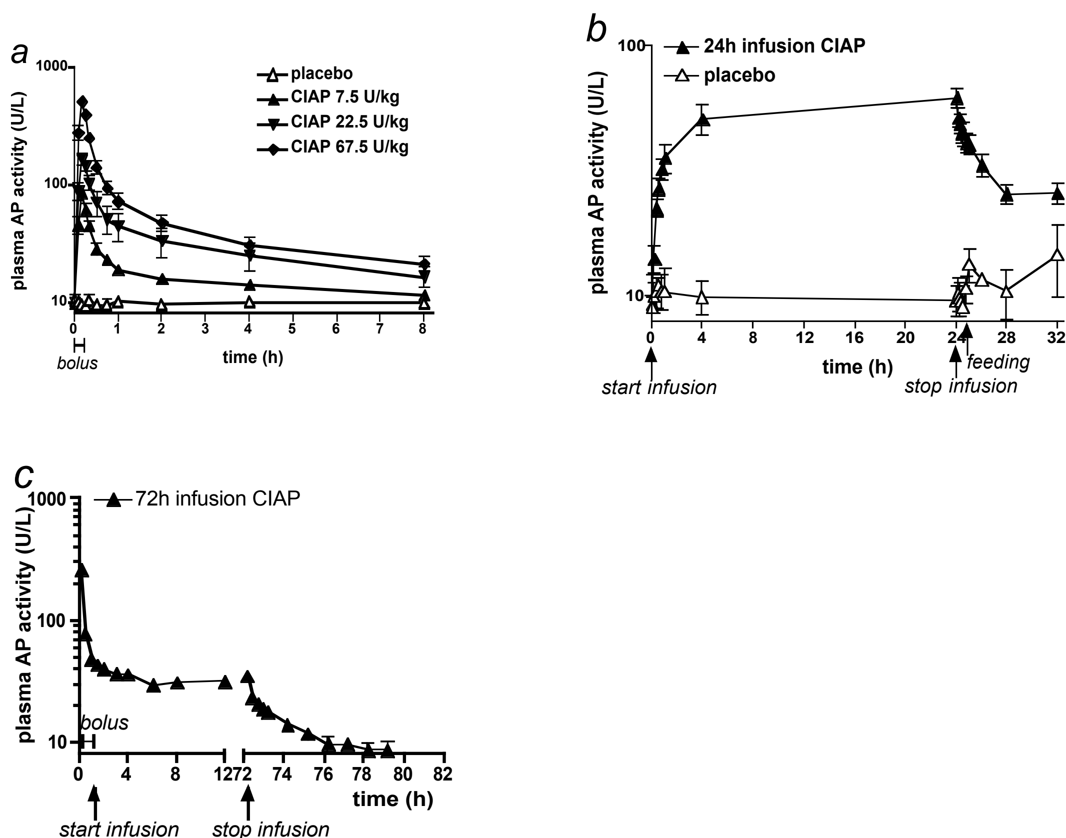

Figure 1 **Pharmacokinetics of BIAP.**

Before, during and after administration of BIAP, alkaline phosphatase activity was measured in serum..
a: 10 min infusion, b: continuous infusion of 200 units BIAP/kg body weight/24 hr and c: 10 min infusion of 67.5 U/kg followed by 72 hr at 177.5 U/kg/day. Depicted are mean ± S.E.M for each dosing group (n=6).

Discussion

Bovine calf intestinal alkaline phosphatase (BIAP), an enzyme isolated from the intestinal mucosa of bovine calves, has previously been demonstrated to detoxify lipopolysaccharide (LPS, endotoxin) *in vivo* [21]. Before BIAP can be applied in the treatment of Gram-negative sepsis, safety, tolerability and the possible induction of specific anti-BIAP antibodies after its administration had to be investigated. Towards this goal, BIAP was administered to various animal species as well as human volunteers as a single intravenous dose or as a multiple-dose regimen (Table I, page 81).

Previously, we showed that treatment of piglets with BIAP for 28 consecutive days did not result in any adverse effects [21]. The present study shows that single dose administration of BIAP did not result in any adverse effects in mice, rats, dogs and humans. Also, treatment according to a multiple-dosing regimen did not lead to any adverse effects in dogs. In contrast, multiple dosing of BIAP to mice resulted in serum sickness. The induction of serum sickness can explain the clinical chemistry results found at interim necropsy (increased plasma creatinin and decreased albumin) and the renal lesions observed in animals sacrificed at day 11. Other changes found in surviving mid- and high-dose mice at interim necropsy that can be attributed to serum sickness include changes in clinical chemistry values (decreases in total protein, albumin/globulin ratio, phospholipids and cholesterol level and increases in urea, triglycerides and glucose level) and organ weights (increases in liver and kidney weights). Changes in chloride levels in low and mid-dose groups were not ascribed to treatment since no dose-response relation was detected and the increase in the low-dose group was not confirmed at day 28. At both interim and final necropsy, animals of the low and mid-dose groups showed severe tail lesions (wounds, encrustations, necrosis or tip of tail missing). These tail lesions may represent a local, immune-mediated phenomenon occurring in sensitized animals (Arthus reaction; [23]).

The finding that BIAP potentially may give rise to antibody formation and sensitization was further explored in a study using guinea pigs. Here, the test substance BIAP caused severe signs of anaphylaxis in both the ip and iv sensitized animals upon a BIAP challenge in the ASA test on day 28. The positive responses in the PCA tests also indicate that sensitized animals did produce BIAP-specific reaginic antibodies.

The combined results of the mice and guinea pig studies clearly show that treatment with BIAP according to a multiple-dosing regimen may induce BIAP-specific antibodies that can cause anaphylactic shock, leading to death within hours after injection. Therefore, the risk of anaphylaxis in other species including humans cannot be excluded. In contrast to the studies performed in mice, BIAP did not induce specific anti-BIAP antibodies in pigs and dogs. One possible cause for this could be the presence of bovine products in their food, which may have made them tolerant to BIAP. For this reason it was anticipated that humans that consume or have consumed bovine products will not show antibody formation either. This was indeed shown by the absence of BIAP-specific antibodies and negative skin prick tests 90 days after treatment. In human volunteers receiving up to 67.5 U BIAP/kg as a bolus or up to 600 U BIAP/kg over a 72 h infusion, only mild

clinical effects, all scored as unrelated or unlikely related to BIAP by an independent medical doctor, were observed. Apart from safety in man, pharmacokinetic parameters of BIAP were determined. Elimination of BIAP from blood in man showed to be dose independent and biphasic. This is in line with the results from a previous study in piglets [21]. The distribution volume (V_d = 0.418 – 1.5 l/kg) showed a substantial distribution of BIAP over a second, yet undefined, compartment, which is characterized by a first phase fast distribution and a much slower second phase clearance. There are several hypotheses that may explain this phenomenon.

First, being a non-sialylated glycoprotein, BIAP is likely to be phagocytosed by hepatocytes using their asialoglycoprotein receptor (ASGPR) [24,25]. This mechanism has been shown to be responsible for the short serum half-life of IAP in the early phase. Indeed, Scholtens *et al.* [26,27] showed that the plasma residence time of canine intestinal AP 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 several hours. Second, BIAP, being an glycosylphosphatidylinositol (GPI)-anchored protein, may spontaneously attach to cell membranes [28,29]. Due to the huge surface of the blood vessel endothelium, we hypothesize that administered BIAP binds to these endothelial cells in the first phase of clearance, resulting in a fast initial disappearance of BIAP from the plasma. In the second phase, release of BIAP from the endothelial wall into the bloodstream would result in much slower second phase plasma elimination.

In conclusion, multiple-dose BIAP administration to different animal species resulted in species-dependent antibody formation and toxic effects. Administration of high doses of BIAP to humans did not result in specific anti-BIAP antibody induction or any acute/subacute toxic effects. Based on these safety studies we therefore conclude that single dose BIAP administration to humans is safe. At present, a multi-centre phase II clinical trial with sepsis patients is ongoing.

APPENDIX

TABLE I Protocol outlines of safety studies with BIAP performed in multiple species.

Species	Route	Dose (U/kg/day)	Days observed following dosing	Clin obs.	Hematology	Clin Chem.	Urinalysis	Macro path.	Immunogenicity
Single-dose									
Mouse	iv	5354	14	Y	N	N	N	Y	N
Rat	iv	2677	14	Y	N	N	N	Y	N
Dog	iv	15, 75 and 750	1	Y	N	N	N	N	N
Multiple-dose									
Mouse	iv	75, 750 and 7500	dosed 28 days	Y	Y	Y	Y	Y	Y
Guinea pig	iv / ip	0.005 and 0.05 mg [a] challenge: 5 mg	5 x sensitization 1 x challenge	Y	N	N	N	N	Y
Dog	iv	15 and 75	dosed 14 days	Y	Y	Y	Y	Y	Y
Phase I									
Human	iv	7.5, 22.5 and 67.5 [b]	90	Y	Y	Y	Y	N	Y
	iv	200 [c], 600 [d]	90	Y	Y	Y	Y	N	Y

[a]: dose in mg/animal
[b]: dosed as a 10 minutes infusion
[c]: dosed over 24 hour infusion
[d]: dosed over 72 hour infusion

Clin. obs.: clinical observations (animal well being).
Clin. chem.: clinical chemistry measurements
Macro path.: macroscopical pathology

TABLE II

Summary of major clinical signs in mice.

The number of animals showing the observation after nine days of BIAP administration.

Group 10 Males / 10 Females	Control		75 U/kg		750 U/kg		7500 U/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
Dead	0	0	0	0	3	2	6	7
Killed (unscheduled)	0	0	0	1	0	0	0	0
Behaviour								
sluggish	0	0	0	0	1	0	6	3
hyperactivity	0	0	0	0	0	1	0	0
Eyes								
blepharospasm	0	0	0	0	1	0	6	3
corneal opacity/keratitis	1	0	0	0	0	0	0	0
cataract	0	1	0	0	0	0	0	0
pale	1	0	0	3	0	0	0	0
Skin/fur								
piloerection	1	0	1	2	1	0	6	3
Tail								
ringtail	0	0	0	0	1	0	0	0
wound	0	0	3	5	0	0	0	0
encrustation	0	0	6	7	6	1	0	0
tip of tail missing	0	0	4	6	2	0	0	0
necrosis	0	0	4	3	0	0	0	0

TABLE III

Clinical chemistry measurements in the multiple dose toxicity study on BIAP in mice.

Mean results in plasma collected at interim necropsy on day 11.

Parameter	MALES			FEMALES		
	Control (n=3)	75 U/kg (n=3)	750 U/kg (n=1)	Control (n=3)	75 U/kg (n=3)	750 U/kg (n=3)
ALP (U/l)	116 ± 25	133 ± 40	156	128 ± 9	58 ± 7	143 ± 48
ALAT (U/l)	61 ± 5	255 ± 171	74	51 ± 9	35 ± 8	50 ± 5
ASAT (U/l)	129 ± 12	247 ± 146	128	125 ± 18	82 ± 9	148 ± 36
GGT (U/l)	0 ± 0	0 ± 0	0	0 ± 0	0 ± 0	0 ± 0
TP (g/l)	54 ± 2	55 ± 1	44	58 ± 2	53 ± 1	43 ± 2 **
Albumin (g/l)	32 ± 1	33 ± 1	24	38 ± 1	31 ± 3	27 ± 2 *
Glucose (mmol/l)	10.41 ± 0.13	9.58 ± 0.67	11.28	8.30 ± 0.58	8.43 ± 0.68	16.74 ± 5.41
A/G ratio	1.46 ± 0.06	1.52 ± 0.16	1.20	1.90 ± 0.0	1.41 ± 0.24	1.61 ± 0.14
Urea (mmol/l)	7.3 ± 1.1	6.8 ± 0.8	13.0	5.9 ± 0.3	4.9 ± 0.5	11.7 ± 1.8 *
Creatinin (µmol/l)	9 ± 1	10 ± 1	18	11 ± 0	9 ± 0	19 ± 1 **
Bilirubin (µmol/l)	0.2 ± 0.2	0.9 ± 0.4	0.0	0.2 ± 0.2	0.4 ± 0.2	0.0 ± 0.0
Cholesterol (mmol/l)	3.68 ± 0.61	3.38 ± 0.24	2.66	2.61 ± 0.11	2.64 ± 0.34	1.74 ± 0.10
Triglycerides (mmol/l)	1.61 ± 0.18	1.35 ± 0.58	1.54	1.35 ± 0.16	1.00 ± 0.12	2.76 ± 0.37 *
Phospholipids(mmol/l)	3.55 ± 0.40	3.44 ± 0.20	2.46	2.60 ± 0.05	2.46 ± 0.29	1.85 ± 0.01 *
Inorganic P (mmol/l)	3.16 ± 0.10	3.09 ± 0.08	3.30	2.86 ± 0.08	3.17 ± 0.19	3.37 ± 0.26
Calcium (mmol/l)	2.57 ± 0.04	2.61 ± 0.02	2.52	2.64 ± 0.05	2.58 ± 0.03	2.53 ± 0.06
Chloride (mmol/l)	114 ± 1	112 ± 1	112	112 ± 0	114 ± 0 *	109 ± 1 **
Potassium (mmol/l)	7.1 ± 0.3	6.7 ± 0.2	7.4	6.2 ± 0.2	6.4 ± 0.3	6.3 ± 0.1
Sodium (mmol/l)	155 ± 1	154 ± 1	160	152 ± 0	154 ± 0	153 ± 1

ALP: alkaline phosphatae

ALAT: alanine aminotransferase

ASAT:: aspartate aminotransferase

GGT: gamma glutamyl transferase

TP: total protein

A/G ratio: albumin/globulin ratio

Values are depicted as means ± SEM.

* p<0.05 and ** p<0.01.

TABLE IV

Mean body weights (g) and relative organ weights (g/kg body weight) at necropsy on day 11 and 28. Values are depicted as means ± SEM. Asterisks denote significant differences between the group injected with BIAP and the control group (*p<0.05, **p<0.01).
--- : not determined.

Parameter	MALES					FEMALES					
	Control day 11	Control day 28	75 U/kg day 11	75 U/kg day 28	750 U/kg day 11	Control day 11	Control day 28	75 U/kg day 11	75 U/kg day 28	750 U/kg day 11	7500 U/kg day 11
Body weight day 0	31.6 ± 0.4	31.6 ± 0.4	31.8 ± 0.5	31.8 ± 0.5	31.8 ± 0.4	25.7 ± 0.4	25.7 ± 0.4	25.8 ± 0.5	25.8 ± 0.5	25.8 ± 0.4	25.8 ± 0.5
Body weight	33.1 ± 0.9	31.8 ± 0.8	32.8 ± 0.8	31.6 ± 0.5	31.4 ± 1.0	25.8 ± 0.3	25.8 ± 0.5	25.9 ± 0.9	26.4 ± 0.7	27.2 ± 1.0	26.0 ± 0.9
Pituitary	0.253 ± 0.059	0.228 ± 0.018	0.213 ± 0.016	0.222 ± 0.009	0.335 ± 0.002	0.245 ± 0.013	0.313 ± 0.054	0.206 ± 0.013	0.272 ± 0.029	0.294 ± 0.018	0.233 ± 0.029
Thyroid	0.292 ± 0.010	0.302 ± 0.013	0.305 ± 0.030	0.248 ± 0.014	0.320 ± 0.017	0.400 ± 0.017	0.445 ± 0.046	0.334 ± 0.018	0.326 ± 0.035	0.322 ± 0.055	0.544 ± 0.145
Adrenals	0.343 ± 0.016	0.436 ± 0.036	0.334 ± 0.023	0.380 ± 0.030	0.402 ± 0.069	0.605 ± 0.049	0.582 ± 0.029	0.563 ± 0.067	0.569 ± 0.025	0.628 ± 0.038	0.646 ± 0.100
Kidneys	16.20 ± 1.18	15.35 ± 0.45	16.29 ± 0.11	16.58 ± 0.38	15.21 ± 1.45	11.58 ± 0.19	12.75 ± 0.39	12.07 ± 0.77	13.25 ± 0.62	12.11 ± 0.30	13.98 ± 0.22 *
Thymus	2.24 ± 0.49	1.03 ± 0.04	1.98 ± 0.15	1.10 ± 0.09	1.47 ± 0.32	2.19 ± 0.11	1.89 ± 0.19	2.13 ± 0.53	1.88 ± 0.12	2.37 ± 0.50	2.26 ± 0.20
Brain	14.08 ± 0.38	14.77 ± 0.30	14.95 ± 0.53	15.18 ± 0.17	14.47 ± 0.35	18.91 ± 0.21	18.47 ± 0.34	18.25 ± 0.42	18.49 ± 0.58	17.94 ± 0.41	18.73 ± 0.66
Spleen	3.41 ± 0.44	4.22 ± 0.38	3.63 ± 0.28	4.51 ± 0.25	2.82 ± 0.18	3.84 ± 0.38	7.43 ± 0.45	6.29 ± 1.56	8.24 ± 0.69	5.04 ± 0.35	5.05 ± 0.50
Heart	5.97 ± 0.74	5.63 ± 0.24	6.43 ± 0.74	6.11 ± 0.24	5.51± 0.35	5.76 ± 0.45	5.84 ± 0.16	6.40 ± 0.76	5.88 ± 0.43	5.21 ± 0.19	6.30 ± 0.59
Liver	52.4 ± 1.3	53.7 ± 0.9	53.9 ± 0.7	51,7 ± 1.7	61.1 ± 4.9	47.3 ± 2.3	46.6 ± 1.5	46.8 ± 2.1	49.3 ± 1.3	55.7 ± 1.5 *	59.9 ± 0.6 **
Lung	7.53 ± 0.40	8.01 ± 0.46	8.22 ± 0.49	7.74 ± 0.48	6.85 ± 0.15	9.25 ± 0.34	7.94 ± 0.25	8.24 ± 0.35	7.84 ± 0.30	7.74 ± 0.34	8.62 ± 0.42
Testes	6.14 ± 0.15	6.81 ± 0.17	6.83 ± 0.88	6.69 ± 0.24	6.07 ± 0.26	---	---	---	---	---	---
Prostate	1.09 ± 0.11	2.61 ± 0.30	1.46 ± 0.09	2.46 ± 0.26	1.53 ± 0.59	---	---	---	---	---	---
Sem. vesicles	7.61 ± 0.79	7.24 ± 0.78	9.39 ± 0.51	7.43 ± 0.49	6.66 ± 0.57	---	---	---	---	---	---
Ovaries	---	---	---	---	---	0.721 ± 0.066	0.793 ± 0.116	0.751 ± 0.080	0.793 ± 0.028	0.726 ± 0.038	0.985 ± 0.198
Uterus	---	---	---	---	---	5.08 ± 1.06	6.00 ± 0.83	5.69 ± 2.17	4.93 ± 0.64	7.35 ± 2.04	8.31 ± 1.78

TABLE V

Clinical chemistry measurements in the multiple dose toxicity study on BIAP in dogs.

Parameter	MALES			FEMALES		
	Control (n=4)	15 U/kg (n=4)	75 U/kg (n=4)	Control (n=4)	15 U/kg (n=4)	75 U/kg (n=4)
ALP (U/l)	103 ± 7	98 ± 7	106 ± 4	103 ± 16	85 ± 3	109 ± 9
ALAT (U/l)	38± 4	27 ± 5	38 ± 2	36 ± 3	35 ± 3	36 ± 3
ASAT (U/l)	29 ± 1	25 ± 2	26 ± 2	26 ± 1	27 ± 1	28 ± 3
GGT (U/l)	2.5 ± 0.2	2.1 ± 0.2	1.8 ± 0.7	1.4 ± 0.6	0.7 ± 0.4	2.5 ± 0.2
TP (g/l)	53 ± 1	54 ± 0	54 ± 1	55 ± 1	54 ± 0	55 ± 2
Albumin (g/l)	31 ± 1	31 ± 0	32 ± 1	33 ± 1	33 ± 0	33 ± 0
Glucose (mmol/l)	6.25 ± 0.20	6.20 ± 0.14	6.50 ± 0.09	6.31 ± 0.09	6.19 ± 0.04	6.24 ± 0.06
A/G ratio	1.44 ± 0.02	1.36 ± 0.06	1.43 ± 0.05	1.49 ± 0.09	1.56 ± 0.05	1.53 ± 0.06
Urea (mmol/l)	4.1 ± 0.2	3.7 ± 0.4	3.2 ± 0.3	4.3 ± 0.2	4.5 ± 0.3	4.2 ± 0.3
Creatinin (µmol/l)	52 ± 2	46 ± 2 *	44 ± 1 *	45 ± 4	52 ± 1	52 ± 2
Bilirubin (µmol/l)	1.3 ± 0.5	0.7 ± 0.3	0.6 ± 0.2	0.5 ± 0.3	0.0 ± 0.0	0.8 ± 0.2
Cholesterol (mmol/l)	5.41 ± 0.44	6.51 ± 0.68	4.87 ± 0.45	4.84 ± 0.33	5.21 ± 0.20	6.32 ± 0.78
Triglycerides (mmol/l)	0.55 ± 0.05	0.46 ± 0.05	0.46 ± 0.05	0.38 ± 0.05	0.45 ± 0.03	0.54 ± 0.06
Phospholipids (mmol/l)	5.44 ± 0.28	6.12 ± 0.47	5.17 ± 0.37	5.16 ± 0.14	5.37 ± 0.07	5.99 ± 0.45
Inorganic P (mmol/l)	2.37 ± 0.05	2.35 ± 0.05	2.43 ± 0.11	2.38 ± 0.03	2.33 ± 0.12	2.27 ± 0.05
Calcium (mmol/l)	2.77 ± 0.02	2.82 ± 0.04	2.84 ± 0.04	2.81 ± 0.03	2.86 ± 0.04	2.88 ± 0.06
Chloride (mmol/l)	110 ± 1	109 ± 0	110 ± 1	109 ± 1	112 ± 1	111 ± 1
Potassium (mmol/l)	4.5 ± 0.1	4.5 ± 0.1	4.2 ± 0.0	4.8 ± 0.1	4.4 ± 0.1	4.6 ± 0.2
Sodium (mmol/l)	147 ± 0	146 ± 1	146 ± 1	146 ± 1	148 ± 1 *	148 ± 0 *

ALP: alkaline phosphatae
ALAT: alanine aminotransferase
ASAT:: aspartate aminotransferase
GGT: gamma glutamyl transferase
TP: total protein
A/G ratio: albumin/globulin ratio

Values are depicted as means ± SEM.
* $p<0.05$ and ** $p<0.01$.

TABLE VI

Hematology parameters in healthy volunteers after BIAP administration.

Mean results in plasma collected at different days. Values are depicted as means ± SEM

--- : not determined.

Treatment	Time	Hemoglobin (g/dl)	RBC ($x10^{12}/L$)	Platelets ($x10^9/L$)	WBC ($x10^9/L$)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
7.5 U/kg	Day -1	14.47 ± 0.79	4.67 ± 0.31	266.5 ± 62.3	8.18 ± 1.05	---	---	---	---	---
	4 hr post-dose	14.65 ± 0.85	4.70 ± 0.41	257.3 ± 63.9	7.18 ± 1.20	59.82 ± 8.81	2.95 ± 2.70	0.17 ± 0.18	30.33 ± 10.27	6.70 ± 1.40
	24 hr post-dose	14.50 ± 0.79	4.68 ± 0.41	266.3 ± 74.1	7.65 ± 1.50	62.55 ± 4.85	3.28 ± 3.10	0.42 ± 0.33	26.63 ± 5.51	7.10 ± 1.85
	Day 14 follow up	14.78 ± 1.35	4.75 ± 0.43	273.2 ± 71.9	6.60 ± 1.58	56.90 ± 5.26	3.10 ± 3.01	0.37 ± 0.10	31.85 ± 5.82	7.77 ± 1.66
22.5 U/kg	Day -1	15.38 ± 1.23	4.88 ± 0.38	255.3 ± 41.2	7.95 ± 1.44	58.80 ± 7.96	2.28 ± 0.80	0.42 ± 0.28	29.78 ± 7.16	8.72 ± 2.24
	4 hr post-dose	15.25 ± 0.64	4.88 ± 0.39	233.8 ± 57.5	6.47 ± 2.21	56.07 ± 14.34	2.43 ± 1.16	0.30 ± 0.25	32.65 ± 12.26	8.55 ± 1.86
	24 hr post-dose	15.37 ± 0.66	4.94 ± 0.33	258.2 ± 38.7	6.50 ± 1.89	56.72 ± 9.82	3.22 ± 1.19	0.33 ± 0.23	31.08 ± 8.71	8.65 ± 0.89
	Day 14 follow up	15.30 ± 1.03	4.93 ± 0.46	278.5 ± 45.0	7.17 ± 3.74	53.42 ± 14.02	4.22 ± 1.82	0.35 ± 0.24	33.60 ± 11.61	8.43 ± 1.85
62.5 U/kg	Day -1	15.05 ± 1.55	4.79 ± 0.49	253.3 ± 33.5	7.48 ± 1.21	49.52 ± 4.58	2.87 ± 1.57	0.30 ± 0.17	40.13 ± 4.45	7.18 ± 1.28
	4 hr post-dose	14.87 ± 1.62	4.91 ± 0.44	245.7 ± 26.3	6.03 ± 0.80	51.12 ± 5.11	2.35 ± 1.28	0.27 ± 0.15	39.60 ± 4.84	6.68 ± 1.14
	24 hr post-dose	15.32 ± 1.76	4.97 ± 0.47	249.7 ± 24.4	6.33 ± 1.29	51.77 ± 10.04	2.72 ± 1.54	0.27 ± 0.27	37.25 ± 9.68	8.00 ± 1.01
	Day 14 follow up	15.08 ± 1.60	4.92 ± 0.52	269.3 ± 35.1	6.40 ± 0.70	50.53 ± 8.23	2.85 ± 1.46	0.23 ± 0.21	38.83 ± 7.68	7.55 ± 2.48
200 U/kg/24 h	Day -1	15.12 ± 0.60	4.92 ± 0.33	298.3 ± 68.3	7.67 ± 0.95	49.85 ± 5.66	2.02 ± 1.22	0.38 ± 0.26	39.58 ± 6.31	8.15 ± 1.95
	4 hr post-dose	15.27 ± 0.72	4.80 ± 0.41	258.8 ± 62.1	6.28 ± 1.82	53.48 ± 8.97	1.93 ± 1.09	0.43 ± 0.16	35.82 ± 8.16	8.33 ± 1.85
	12 hr post-dose	14.88 ± 1.01	4.80 ± 0.43	255.5 ± 86.0	7.07 ± 1.75	57.52 ± 10.48	1.87 ± 1.07	0.67 ± 0.66	34.33 ± 9.49	5.62 ± 1.01
	24 hr post-dose	14.77 ± 0.53	4.81 ± 0.42	254.7 ± 55.9	5.82 ± 0.81	---	---	---	---	---
	32 hr post-dose	14.58 ± 0.78	4.74 ± 0.42	222.0 ± 102	6.55 ± 0.94	54.88 ± 6.56	2.05 ± 1.17	0.42 ± 0.17	34.72 ± 6.12	7.92 ± 2.58
	Day 14 follow up	15.35 ± 0.52	4.96 ± 0.34	275.8 ± 40.7	6.65 ± 1.37	55.27 ± 11.72	2.15 ± 1.22	0.65 ± 0.54	32.42 ± 11.36	9.48 ± 3.05
600 U/kg/72 h	Day -1	15.34 ± 1.03	4.91 ± 0.39	239.0 ± 29.7	7.58 ± 1.36	60.38 ± 2.63	2.08 ± 1.72	0.40 ± 0.24	30.24 ± 3.95	6.90 ± 0.56
	4 hr post-dose	14.72 ± 0.90	4.87 ± 0.37	212.6 ± 26.0	5.46 ± 1.29	54.60 ± 8.36	1.92 ± 1.82	0.34 ± 0.45	34.34 ± 9.46	7.78 ± 1.06
	24 hr post-dose	15.36 ± 0.75	5.02 ± 0.15	226.2 ± 34.7	4.60 ± 1.33	58.60 ± 8.48	2.86 ± 1.12	0.46 ± 0.21	30.48 ± 8.56	7.58 ± 1.60
	72 hr post-dose	14.92 ± 0.95	4.69 ± 0.35	190.6 ± 30.4	4.52 ± 0.91	55.40 ± 7.25	2.86 ± 0.56	0.44 ± 0.23	32.60 ± 7.51	8.64 ± 0.56
	Day 14 follow up	14.78 ± 0.77	4.83 ± 0.33	255.8 ± 53.8	5.40 ± 1.92	54.40 ± 9.72	2.86 ± 0.53	0.38 ± 0.16	34.04 ± 9.81	8.36 ± 0.53

TABLE VII

Clinical chemistry parameters in healthy volunteers after administration of BIAP.

Mean results in plasma collected at different days.

7.5 U/kg	Day -1	4 hr post-dose	24 hr post-dose	Day 14 follow up
ALP (U/l)	64.8 ± 8.2	70.2 ± 13.9	62.7 ± 8.8	---
ALAT (U/l)	28.2 ± 6.5	30.0 ± 7.6	27.5 ± 5.8	33.8 ± 7.8
ASAT (U/l)	22.0 ± 3.2	23.5 ± 6.3	18.0 ± 3.0	25.8 ± 7.4
GGT (U/l)	6.025.0 ± 6.3	23.8 ± 6.3	24.2 ± 6.2	25.5 ± 6.0
TP (g/l)	72.3 ± 4.8	72.0 ± 2.6	70.7 ± 4.2	72.7 ± 3.4
Albumin (g/l)	40.0 ± 3.3	39.7 ± 2.3	39.0 ± 1.8	46.0 ± 2.7
Glucose (mg/dl)	86.7 ± 4.3	80.0 ± 5.1	90.3 ± 3.4	86.3 ± 5.4
Urea (mg/dl)	30.3 ± 7.1	31.2 ± 5.5	39.7 ± 6.1	33.7 ± 8.6
Creatinin (mg/dl)	0.94 ± 0.15	0.90 ± 0.13	1.02 ± 0.15	0.95 ± 0.12
Bilirubin (mg/dl)	0.7 ± 0.2	1.0 ± 0.6	0.9 ± 0.3	0.8 ± 0.4
Cholesterol (mg/dl)	185 ± 30	185 ± 29	188 ± 30	204 ± 40
Triglycerides (mg/dl)	92.2 ± 23.9	92.2 ± 34.8	114.3 ± 39.6	116.7 ± 41.7
Inorganic P (mmol/l)	1.17 ± 0.15	1.13 ± 0.13	1.08 ± 0.19	1.12 ± 0.11
Calcium (meq/l)	4.58 ±0.19	4.62 ± 0 .10	4.72 ± 0.17	4.73 ± 0.15
Potassium (meq/l)	4.15 ± 0.16	4.72 ± 0.62	4.20 ± 0.25	4.20 ± 0.29
Sodium (meq/l)	139.5 ± 0.5	140.0 ± 1.3	141.3 ± 0.8	141.7 ± 1.4

22.5 U/kg	Day -1	4 hr post-dose	24 hr post-dose	Day 14 follow up
ALP (U/l)	69.5 ± 15.0	101.2 ± 38.0	69.3 ± 12.6	---
ALAT (U/l)	35.7 ± 8.9	37.5 ± 8.1	32.5 ± 8.9	36.0 ± 10.9
ASAT (U/l)	25.2 ± 5.2	22.8 ± 3.7	21.3 ± 3.6	25.8 ± 4.4
GGT (U/l)	25.3 ± 3.2	25.0 ± 4.7	24.3 ±4.6	27.3 ± 6.9
TP (g/l)	77.2 ± 4.8	71.0 ± 3.8	64.2 ± 2.3	71.7 ± 3.6
Albumin (g/l)	43.0 ± 2.4	41.3 ± 2.5	38.0 ± 1.5	41.7 ± 2.2
Glucose (mg/dl)	84.2 ± 4.8	87.8 ± 6.0	85.7 ± 4.7	84.3 ± 16.5
Urea (mg/dl)	30.3 ± 3.9	31.0 ± 5.3	35.5 ± 4.4	31.2 ± 5.3
Creatinin (mg/dl)	0.89 ± 0.08	0.85 ± 0.06	1.02 ± 0.09	0.91 ± 0.08
Bilirubin (mg/dl)	0..8 ± 0.5	1.3 ± 0.6	1.2 ± 0.6	0.7 ± 0.2
Cholesterol (mg/dl)	150 ± 26	148 ± 40	143 ± 32	153 ± 29
Triglycerides (mg/dl)	78.7 ± 32.4	83.3 ± 54.4	103.0 ± 58.0	95.0 ± 66.6
Inorganic P (mmol/l)	1.39 ± 0.16	1.25 ± 0.10	1.00 ± 0.18	1.17 ± 0.18
Calcium (meq/l)	4.65 ± 015	4.60 ± 0.17	4.55 ± 0.14	4.52 ± 0.10
Potassium (meq/l)	4.18 ± 0.20	4.10 ± 0.28	4.35 ± 0.30	4.07 ± 0.25
Sodium (meq/l)	140.7 ± 0.5	139.8 ± 1.0	141.5 ± 0.5	143.2 ± 1.2

62.5 U/kg	Day -1	4 hr post-dose	24 hr post-dose	Day 14 follow up
ALP (U/l)	68.5 ± 20.8	129.2 ± 36.1	75.8 ± 18.4	69.0 ± 15.9
ALAT (U/l)	29.2 ± 4.2	33.3 ± 4.5	29.3 ± 5.3	31.2 ± 10.1
ASAT (U/l)	24.0 ± 7.7	22.0 ± 5.1	19.5 ± 2.6	24.8 ± 3.7
GGT (U/l)	24.7 ± 7.7	24.8 ± 7.3	24.5 ± 6.8	25.7 ± 9.0
TP (g/l)	64.8 ± 2.5	68.3 ± 2.4	63.3 ± 3.3	73.3 ± 2.7
Albumin (g/l)	40.2 ± 2.6	40.7 ± 1.9	39.0 ± 1.8	41.8 ± 2.3
Glucose (mg/dl)	81.5 ± 2.9	83.5 ± 3.6	87.0 ± 5.6	85.3 ± 6.2
Urea (mg/dl)	32.3 ± 6.6	29.8 ± 3.2	31.7 ± 3.1	29.5 ± 2.7
Creatinin (mg/dl)	0.97 ± 0.12	0.89 ± 0.11	1.01 ± 0.10	0.96 ± 0.08
Bilirubin (mg/dl)	0.6 ± 0.2	0.9 ± 0.3	0.7 ± 0.2	0.7 ± 0.3
Cholesterol (mg/dl)	172 ± 52	176 ± 52	176 ± 53	180 ± 51
Triglycerides (mg/dl)	109.3 ± 38.1	96.3 ± 27.0	113.5 ± 29.3	116.5 ± 50.9
Inorganic P (mmol/l)	1.21 ± 0.06	1.18 ± 0.06	0.97 ± 0.06	1.06 ± 0.05
Calcium (meq/l)	4.50 ± 0.11	4.60 ± 0.09	4.38 ± 0.08	4.45 ± 0.18
Potassium (meq/l)	4.12 ± 0.15	4.13 ± 0.05	4.15 ± 0.14	4.25 ± 0.33
Sodium (meq/l)	142.3 ± 1.5	139.3 ± 1.2	141.3 ± 1.4	143.0 ± 1.9

200 U/kg/24 h	Day -1	4 hr post-dose	12 hr post-dose	24 hr post-dose	32 hr post-dose	Day 14 follow up
ALP (U/l)	67.3 ± 10.4	---	151.0 ± 40.3	175.0 ± 28.0	71.3 ± 7.7	70.2 ± 11.1
ALAT (U/l)	32.8 ± 9.9	29.7 ± 7.2	24.3 ± 4.0	27.7 ± 6.7	24.3 ± 6.2	38.8 ± 19.2
ASAT (U/l)	24.7 ± 9.4	21.0 ± 6.4	19.2 ± 5.4	19.5 ± 6.0	31.3 ± 19.0	24.5 ± 9.3
GGT (U/l)	27.7 ± 5.2	26.8 ± 5.4	25.7 ± 5.0	26.0 ± 4.3	24.0 ± 4.1	27.8 ± 5.6
TP (g/l)	73.5 ± 4.0	70.3 ± 2.9	67.5 ± 4.2	67.3 ± 3.3	71.8 ± 4.2	74.3 ± 3.6
Albumin (g/l)	41.5 ± 3.5	39.2 ± 1.6	37.0 ± 2.8	37.8 ± 1.5	40.3 ± 3.6	41.3 ± 2.9
Glucose (mg/dl)	79.0 ± 6.8	74.3 ± 5.2	141.7 ± 17.1	83.2 ± 3.1	85.3 ± 14.6	81.5 ± 6.3
Urea (mg/dl)	26.2 ± 9.0	25.0 ± 4.4	34.7 ± 3.1	29.5 ± 3.4	36.2 ± 4.3	23.5 ± 7.7
Creatinin (mg/dl)	01.00 ± 0.11	0.90 ± 0.10	0.90 ± 0.12	0.92 ± 0.10	0.94 ± 0.12	0.98 ± 0.12
Bilirubin (mg/dl)	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.2	0.8 ± 0.5	0.5 ± 0.2
Cholesterol (mg/dl)	178 ± 35	176 ± 40	170 ± 38	174 ± 37	177 ± 37	177 ± 37
Triglycerides (mg/dl)	152.8 ± 77.3	105.3 ± 44.0	153.2 ± 67.8	133.5 ± 55.2	253.3 ± 96.6	111.7 ± 35.1
Inorganic P (mmol/l)	1.15 ± 0.25	1.01 ± 0.13	1.04 ± 0.11	0.98 ± 0.15	1.23 ± 0.12	0.98 ± 0.11
Calcium (meq/l)	4.48 ± 0.12	4.42 ± 0.04	4.40 ± 0.09	4.43 ± 0.08	4.42 ± 0.10	4.50 ± 0.22
Potassium (meq/l)	4.25 ± 0.26	4.22 ± 0.26	3.68 ± 0.23	4.35 ± 0.16	4.93 ± 1.16	4.53 ± 0.49
Sodium (meq/l)	143.5 ± 1.5	142.0 ± 1.8	141.2 ± 1.0	141.3 ± 1.6	141.3 ± 1.2	142.8 ± 2.1

600 U/kg/72 h	Day -1	4 hr post-dose	24 hr post-dose	72 hr post-dose	Day 14 follow up
ALP (U/l)	79.80 ± 25.55	160.60 ± 25.05	153.00 ± 25.96	162.00 ± 23.65	70.60 ± 21.55
ALAT (U/l)	46.80 ± 23.47	42.40 ± 24.27	46.40 ± 29.83	47.40 ± 27.84	38.80 ± 10.73
ASAT (U/l)	33.40 ± 6.03	28.80 ± 8.11	29.20 ± 10.28	31.80 ± 10.23	33.00 ± 11.07
GGT (U/l)	33.40 ± 18.45	30.60 ± 18.08	32.00 ± 19.04	32.40 ± 19.23	30.60 ± 16.94
TP (g/l)	78.40 ± 5.90	69.00 ± 3.00	72.40 ± 6.23	77.20 ± 5.31	71.40 ± 3.78
Albumin (g/l)	46.40 ± 4.16	39.40 ± 1.52	42.80 ± 4.97	45.40 ± 4.22	44.20 ± 2.17
Glucose (mg/dl)	76.6 ± 5.0	74.2 ± 4.44	78.0 ± 22.8	73.4 ± 4.4	83.0 ± 8.5
Urea (mg/dl)	31.2 ± 5.7	28.4 ± 5.2	33.0 ± 5.8	31.8 ± 1.8	30.2 ± 3.6
Creatinin (mg/dl)	1.04 ± 0.09	0.95 ± 0.06	1.01 ± 0.04	0.98 ± 0.05	1.09 ± 0.05
Bilirubin (mg/dl)	0.66 ± 0.31	0.72 ± 0.28	0.58 ± 0.19	0.60 ± 0.14	0.48 ± 0.13
Cholesterol (mg/dl)	198.60 ± 29.97	183.00 ± 30.98	195.00 ± 39.99	201.40 ± 28.61	187.80 ± 23.82
Triglycerides (mg/dl)	143.40 ± 71.66	132.80 ± 71.19	163.60 ± 95.49	138.60 ± 59.19	155.80 ± 59.92
Inorganic P (mmol/l)	1.14 ± 0.10	1.00 ± 0.09	0.95 ± 0.11	1.00 ± 0.11	1.16 ± 0.28
Calcium (meq/l)	4.70 ± 0.21	4.60 ± 0.16	4.56 ± 0.25	4.62 ± 0.18	4.62 ± 0.18
Potassium (meq/l)	4.28 ± 0.28	4.30 ± 0.26	4.62 ± 0.25	4.48 ± 0.21	4.50 ± 0.42
Sodium (meq/l)	141.4 ± 1.95	140.4 ± 1.52	141.8 ± 1.30	142.0 ± 0.71	142.8 ± 0.84

ALP: alkaline phosphatase
ALAT: alanine aminotransferase
ASAT: aspartate aminotransferase
GGT: gamma glutamyl transferase
TP: total protein

Values are depicted as means ± SEM
--- : not determined

TABLE VIII

Pharmacokinetic parameters in healthy volunteers after intravenous administration of BIAP.

Treatment group	C_{max} (U/L)[1]	$AUC_{0-\infty}$ (Uxh/L)[2]	$t_{1/2}$ (h)[3]	CL (L/h/kg)[4]	V_d (L/kg)[5]
7.5 U BIAP/kg	72.8 (16.6)	73.0 (37.8)	3.6 (1.68-5.71)	0.123 (0.055)	0.418 (0.259-0.610)
22.5 U BIAP/kg	155 (43)	218 (196)	4.22 (2.41-6.48)	0.146 (0.061)	0.434 (0.228-1.21)
67.5 U BIAP/kg	519 (68.8)	398 (125)	2.99 (1.72-4.66)	0.189 (0.048)	0.495 (0.275-0.748)
200.0 U BIAP/kg/24 hr	52.9 (14.9)	129 (35.3)	3.77 (3.30-7.90)	0.164 (0.0367)	0.621 (0.302-1.05)
600.0 U BIAP/kg/72 hr	264 (32.6)	2630 (433)	3.54 (2.25-4.65)	0.233 (0.0362)	1.150 (0.898-1.54)

[1] Maximum concentration of alkaline phosphatase: mean (SD)
[2] Area under the curve from t=0 to indefinite: mean (SD)
[3] plasma half-life time: mean (min – max)
[4] clearance from the plasma: mean (SD)
[5] distribution volume: mean (min – max)

References

1 Fishman WH. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

2 McComb RB, Bowers GN, Posen S. Alkaline phosphatases. New York: Plenum Press, 1979

3 Narisawa S, Wennberg C, Millan JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. J Pathol 2001; 193:125-133

4 Beckman G, Beckman L, Holm S, et al. Placental alkaline phosphatase types and transplacental IgG transport. Hum Hered 1995; 45:1-5

5 Beckman G, Beckman L, Magnusson SS. Placental alkaline phosphatase phenotypes and pre-natal selection. Evidence from studies of spontaneous and induced abortions. Hum Hered 1972; 22:473-480

6 Beckman L, Beckman G, Mi MP. The relation between human placental alkaline phosphatase types and some perinatal factors. Hum Hered 1969; 19:258-263

7 Beckman L, Bjorling G, Christodoulou C. Pregnancy enzymes and placental polymorphism. I. Alkaline phosphatse. Acta Genet Stat Med 1966; 16:59-73

8 Van Hoof VO, and De Broe. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. crit. rev. clin. lab. sci. 1994; 31:197-293

9 Flock E, Bollman J. Alkaline phosphatase in the intestinal lymph of the rat. J.Biol.Chem. 1948; 175:439-449

10 Mahmood A, Yamagishi F, Eliakim R, et al. A possible role for rat intestinal surfactant-like particles in transepithelial triacylglycerol transport. J Clin Invest 1994; 93:70-80

11 Poelstra K, Bakker WW, Klok PA, et al. A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Invest 1997; 76:319-327

12 Rietschel ET, Wagner H. Pathology of septic shock: Springer-Verlag Berlin Heidelberg, 1996

13 Barton GM, Medzhitov R. Control of adaptive immune responses by Toll-like receptors. curr opin immunol 2002; 14:380-383

14 Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001; 1:135-145

15 Dickneite G, Leithauser B. Influence of antithrombin III on coagulation and inflammation in porcine septic shock. Arterioscler Thromb Vasc Biol 1999; 19:1566-1572

16 Howe LM. Novel agents in the therapy of endotoxic shock. Expert Opin Investig Drugs 2000; 9:1363-1372

17 Lin E, Lowry SF. The human response to endotoxin. Sepsis 1998; 2:255-262

18 Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001; 344:699-709

19 Poelstra K, Bakker WW, Klok PA, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

20 Bentala H, Verweij WR, Huizinga-Van der Vlag A, et al. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566

21 Beumer C, Wulferink M, Raaben W, et al. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. J Pharmacol Exp Ther 2003; 307:737-744

22 Lieverse AG, van Essen GG, Beukeveld GJ, et al. Familial increased serum intestinal alkaline phosphatase: a new variant associated with Gilbert's syndrome. J Clin Pathol 1990; 43:125-128

23 Janeway CA, Travers P, Walport M, et al. IMMUNOBIOLOGY. The immune system in health and disease. 4 ed: Current Biology Publications, 1999

24 Pricer WE, Jr., Ashwell G. The binding of desialylated glycoproteins by plasma membranes of rat liver. J Biol Chem 1971; 246:4825-4833

25 Pricer WE, Jr., Hudgin RL, Ashwell G, et al. A membrane receptor protein for asialoglycoproteins. Methods Enzymol 1974; 34:688-691

26 Scholtens HB, Hardonk MJ, Meijer DK. A kinetic study of hepatic uptake of canine intestinal alkaline phosphatase in the rat. Liver 1982; 2:1-13

27 Scholtens HB, Meijer DK, Hardonk MJ. A histochemical study on the distribution of injected canine intestinal alkaline phosphatase in rat liver. Liver 1982; 2:14-21

28 Low MG, Zilversmit DB. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918

29 Medof ME, Nagarajan S, Tykocinski ML. Cell-surface engineering with GPI-anchored proteins. Faseb J 1996; 10:574-586

5

Tolerance induction to bovine calf intestinal alkaline phosphatase (BIAP) prevents immunotoxicity after intravenous BIAP treatment in mice

In preparation

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Abstract

It has previously been demonstrated that bovine calf intestinal alkaline phosphatase (BIAP) attenuates the lipopolysaccharide (LPS)-mediated inflammatory response in mice and piglets and might therefore represent a novel therapeutic drug in the treatment of LPS-mediated diseases like Gram-negative sepsis. Safety studies performed in multiple species demonstrated that single dose BIAP administration is safe. Although BIAP administration according to a multiple-dose regimen did not result in any adverse effects in piglets and dogs, mice were prone to anaphylactic shock.

In the present study it was investigated if this species difference in immune reactivity towards BIAP is due to a lack of tolerance to bovine proteins in mice. Since mice do not consume bovine products with their food whereas other species used in the safety studies do, oral tolerance may explain the species differences in immunogenicity. In order to induce tolerance, mice were given BIAP in their drinking water for 20 consecutive days. Thereafter, an immunization protocol was used consisting of daily iv administration of BIAP. The level of tolerance was read by the inability to mount a BIAP-specific antibody response.

Mice that had received long-term oral BIAP administration showed decreased levels of BIAP-specific antibodies after immunization when compared to orally untreated mice. Furthermore, orally BIAP treated mice did not develop anaphylactic shock reactions whereas orally placebo treated mice did. We therefore conclude that oral administration of BIAP to mice resulted in tolerization, which was characterized by the absence of clinically relevant toxicity and decreased levels of BIAP-specific antibodies upon intravenous BIAP administration.

Introduction

Alkaline phosphatases are dimeric membrane-bound ectoenzymes common to most organisms and can be found in many tissues throughout the body. In mammals, four isoforms are distinguished: placental (PLAP), germ cell (GCAP) and intestinal (IAP), being tissue-specific isoenzymes, and the bone-liver-kidney (BLK) isoenzyme, which is also called tissue non-specific [1]. Alkaline phosphatases catalyze the hydrolysis of phosphomonoesters with release of anorganic phosphate at alkaline pH *in vitro* [2] and have been ascribed several biological functions [3-10].

In 1997, it was shown that human placental alkaline phosphatase (HPLAP) dephosphorylates and thereby detoxifies LPS at physiological pH levels [11]. It was also shown that HPLAP reduces mortality in mice lethally infected with Gram-negative bacteria, thereby suggesting a role for alkaline phosphatase in protection against endotoxin insult typical for Gram-negative bacteria [12,13]. More recently, we have demonstrated that bovine calf intestinal alkaline phosphatase (BIAP), like HPLAP, attenuates LPS-induced TNFα levels and hematological changes in piglets and reduces mortality in mice after a lethal *E. coli* infection [14]. Based on these results it was concluded that BIAP could represent a novel therapeutic drug for the treatment of Gram-negative sepsis and other LPS-mediated diseases. In a previous safety study it was demonstrated that the immunogenic potential of BIAP depends on the species used (Beumer *et al.,* in preparation). Multiple intravenous dosing of BIAP resulted in anaphylaxis in mice and guinea pigs, but not in piglets and dogs. In mice, renal lesions were observed as well. A hypothesis that may explain the species differences in immunotoxicity might be the presence of bovine products in the food of piglets and dogs, which may have made them tolerant to BIAP.

The diet of most animals contains a wide variety of proteins of animal or vegetable origin, most of which are potentially antigenic. However, when protein antigens are administered orally, antigen-specific non-responsiveness (also known as oral tolerance) is induced [15].

The present study was initiated to test whether the immunotoxicity of BIAP observed in earlier safety studies in mice and guinea pigs could be explained by a lack of (oral) tolerance. First, the antibody response after multiple intravenous BIAP dosing was studied. Thereafter it was studied if drinking water supplemented with BIAP would induce tolerance and prevent anaphylaxis in mice after multiple BIAP challenge. The results of this study clearly show that oral administration of BIAP can prevent BIAP-specific antibody induction and anaphylaxis in mice after multiple intravenous BIAP challenge.

Materials and methods

Test material, chemicals and reagents

Bovine calf intestinal alkaline phosphatase (BIAP; GMP-processed, purity ALPIXG: 675 U/mg) was from Biozyme (Blaenavon, UK).C3c-complement/FITC was from Dako (Glostrup, Denmark). Purified rat anti-mouse IgE (clone R35-72) was obtained from Pharmingen (San Diego, USA). Purified goat anti mouse IgG1 was from Southern Biotech Ass Inc. (Birmingham, AL, USA). Immuno Fluore Mounting Medium was from MP Biomedicals Inc (Aurora, Ohio) and p-nitrophenylphosphate was from Acros (Geel, Belgium). Costar high-binding ELISA plates were from Corning B.V. Life Sciences (Schiphol-Rijk, The Netherlands).

Animal study design

Animals

Female BALB/c mice (8-9 week old) were obtained from Charles River (The Netherlands) and housed at the animal facilities (Gemeenschappelijk Dierenlaboratorium, GDL) of Utrecht University. Mice were kept under conventional conditions in filter-topped macrolon cages (four to six mice per cage) at a temperature of 22 ± 3˚C and a 12 h light/dark cycle. Animals received SDS D3 food pellets and acidified drinking water *ad libitum*. Mice were randomly assigned to the study groups and allowed to acclimatize to the laboratory conditions for 13 days before the start of the experiments.

Treatment

All experiments were conducted according to the guidelines of the Animals Ethics Committee of the Faculty of Veterinary Medicin, Utrecht University (Utrecht, The Netherlands).

Intravenous toxicity study with BIAP

BIAP was diluted in sterile 0.9% saline and administered in the tail vein at 75, 200 or 750 U/kg body weight once daily for 28 consecutive days. Up to two hours after dosing, mice were observed for signs of toxicity. When signs of toxicity occurred, animals were observed for a longer period of time until signs disappeared. Body weight, and food and water consumption were recorded daily. Body temperature was assessed daily until day 11, thereafter it was checked twice a week. At necropsy (days 10 and 28) blood was drawn by heart puncture, after which sera were prepared and screened for BIAP specific IgE and IgG1. Liver, lung, kidney and spleen were preserved for immuno-histopathological examination.

Oral tolerance study with BIAP

Mice in the oral BIAP groups (four mice per cage) were given non-acidified drinking water supplemented with BIAP at a concentration of 1 mg BIAP/animal/day for 20 consecutive days. Since in a previous study analysis of drinking water consumption resulted in 4 ml/animal/day, 5 mg BIAP was dissolved in 20 ml drinking water. Stability tests showed that enzymatic activity of BIAP in drinking water decreased with a maximum of 10% within 24 hours after preparation. Oral placebo groups received non-acidified drinking water alone. Drinking water of both groups was replaced every day. After this 20-day period, animals were iv challenged once daily for 11 consecutive days. For this purpose, BIAP was diluted in sterile 0.9% saline and administered in the tail vein at 200 or 750 U/kg bodyweight. Iv placebo group animals received saline only. Up to two hours after dosing, mice were observed for signs of anaphylaxis. Body weight, and food and water consumption were recorded daily. Body temperature was assessed daily until the last day of treatment (day 11). At necropsy (day 14), blood was drawn by heart puncture, after which sera were prepared and screened for BIAP specific IgG1.

Methods

(Immuno)-histopathological examination

Samples of liver, kidney and spleen were preserved in formalin (4 % in PBS). Also, part of kidney, spleen and lungs were snap-frozen in liquid nitrogen for future immunohistological analysis. Lungs were carefully filled with a solution of 1.5% (w/v) glycerine in PBS to expand the vacuoles and allow for optimal preparation of organ sections. Kidneys were also embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Periodic Acid Schiff (PAS) staining was also performed. Since in a previous safety study with multiple dose BIAP in mice the kidney was found the target organ, histopathological examination was restricted to the kidneys. All other tissues were stored to allow future analysis when necessary.

Analysis of immune complex formation in kidneys

Cryopreserved kidneys were sectioned at 7 μm. From each kidney 3 different sections, 80-100 μm apart, were stained with C3c-complement/FITC for 1 hour at room temperature. After being rinsed with PBS and covered with anti-fading Immuno Fluore Mounting Medium, slides were visualized by fluorescence microscopy and screened for complement deposits.

Analysis of BIAP specific IgE and IgG1 antibodies

BIAP-specific antibodies were measured in serum obtained at day 10 and 28 in the immunotoxicity study and day 14 in the oral tolerance study. The assay was performed in Costar high-binding ELISA plates coated overnight at 4 ± 2°C with 100 μl carbonate buffer containing either 2 μg/ml purified rat anti-mouse IgE or 2 μg/ml purified goat anti mouse IgG1. Washing steps were performed with TBS/0.05%Tween (TBST). After blocking with 150 μl TBST/BSA for 2 hours,

plates were washed and sera (diluted in TBST/BSA; first dilution = 2^3) were added and incubated for 1 h at 37 ± 2°C. After washing, plates were incubated with 100 µl TBST/BSA containing
10 µg/ml BIAP. Finally, plates were washed 3 times with TBST and 1 time with diethanolamine buffer. 100 µl alkaline phosphatase substrate [pNPP, 1 mg/ml in diethanolamine buffer (9.7 % diethanolamine (v/v) and 0.02 % $MgCl_2$ (w/v) in distilled water, pH 9.8)] was added per well. After 30 minutes, the reaction was stopped using 50 µl of a 10% (w/v) EDTA solution and plates were read at 405 nm on a microplate reader. Samples with an OD
smaller than the OD measured for the first sample dilution (2^3) were below the detection limit. Samples with an OD higher than the OD measured for the blanc sample + 3 x SD were designated "positive".

Statistical analysis of the results

Statistical analysis on log-transformed anti-alkaline phosphatase antibody titers was performed using one-way ANOVA with Dunnet post-test (Prism 3.00, Graphpad Software Inc.). Data were considered significant when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).

Results

Immunotoxicity study with BIAP

As demonstrated in a previous safety study (Beumer *et al.*, in preparation), anaphylaxis and renal lesions were observed in mice intravenously given BIAP doses of 750 and 7500 U/kg body weight (bw) once dailly for 10 consecutive days. In contrast, animals once daily dosed with 75 U/kg bw for 28 consecutive days did not show any sign of immunotoxicity. In the present study the safety experiments with mice were repeated in order to investigate the antibody response after multiple intravenous BIAP dosing. For this purpose, animals were once daily intravenously dosed with BIAP doses of 75, 200 or 750 U/kg, or saline for 28 consecutive days followed by observation for clinical signs of toxicity and antibody determination in serum.

On day nine of the study, intravenous administration of high-dose BIAP (750 U/kg bw) resulted in apathy, cyanosis, and shortness of breath in all five animals of this dose group. These anaphylactic reactions started approximately 20 - 30 min after treatment and disappeared approximately 1 hour after the symptoms started. On day 10 of the study, again all animals of the high-dose group suffered from severe anaphylactic reactions. For animal welfare reasons, high-dose group animals were not treated any further whereas treatment of control, low-dose (75 U/kg bw) and mid-dose (200 U/kg bw) animals was continued. Intravenous administration of low- or mid-dose BIAP for 28 consecutive days did not result in any behavioural changes. No differences were observed in bodyweight gain, body temperature and food and water consumption in all animals during the entire study (data not shown).

High titers of BIAP-specific IgG1 were found on day 10 in all mice of the low-dose and high-dose groups and in one out of five mice of the mid-dose group after multiple iv dosing with

BIAP (see Figure 1a). In control animals (placebo), no BIAP-specific IgG1 was detected. On day 28 (see Figure 1b), BIAP-specific IgG1 levels were further increased in the low- and mid-dose groups (continued iv exposure to BIAP), but not in the high-dose group (discontinued iv BIAP exposure). In addition, low BIAP-specific IgE titers were present in 4 out of 10 mice of the low-dose group, 3 out of 10 mice of the mid-dose group and 1 out of 10 mice of the high-dose group after iv BIAP administration. In control animals (placebo), no BIAP-specific IgG1 or IgE was detected. Figure 1 also shows that BIAP administration on the day of sacrifice does not interfere with the presence of circulating antibodies.

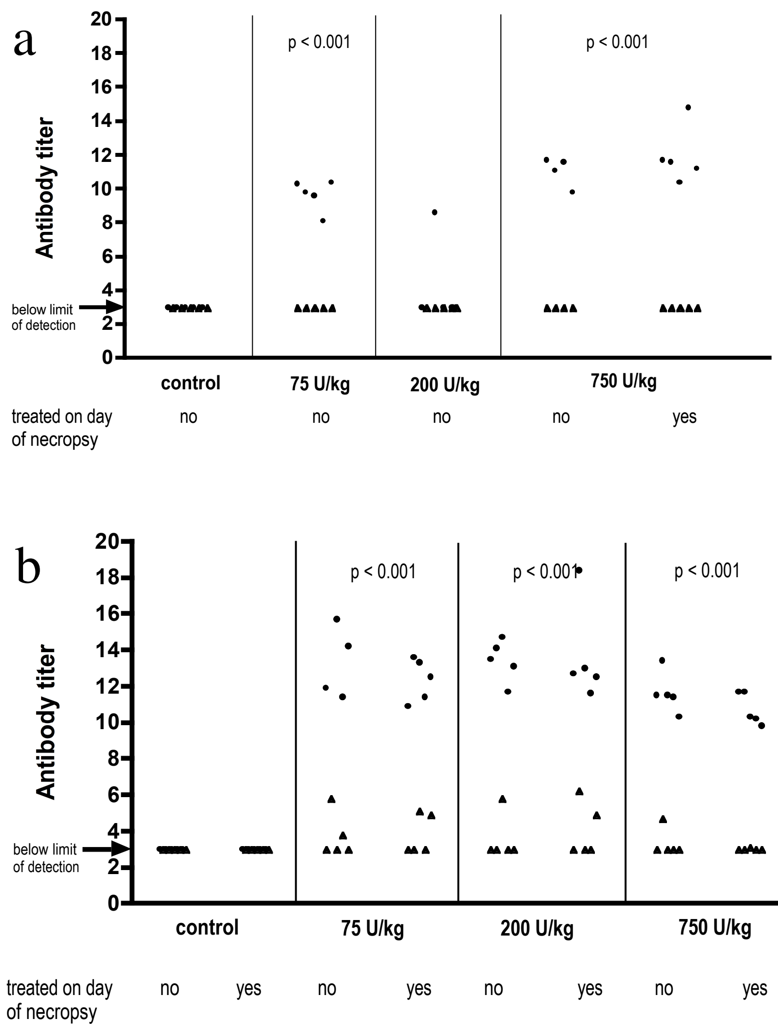

Figure 1: BIAP specific IgG1 (dots) and IgE (triangles) titers in serum after 10 (a) or 28 (b) days of i.v. BIAP treatment. Limit of detection equals no measurable IgG1 or IgE in the first dilution (2^3 = 1:8) tested. Data of individual mice are depicted. P-values denote statistical significance in IgG1 titers of BIAP-treated groups in comparison to the control group.

Gross examination at necropsy did not reveal macroscopic pathological changes in any of the test and control animals. No histopathological changes were observed in HE- and PAS-stained paraffin sections of the kidneys. Frozen kidney sections stained with C3c-complement/FITC showed that multiple intravenously dosing with BIAP did not result in complement deposits in the kidneys.

Oral tolerance study with BIAP

This study was developed to investigate if drinking water supplemented with BIAP would induce tolerance and thereby prevent anaphylaxis in mice after multiple BIAP challenge. For this purpose, animals were given BIAP in their drinking water or drinking water alone for 20 consecutive days. After this period, mice were once daily intravenously challenged with 0, 200 or 750 U BIAP/kg bw for 11 consecutive days.

Placebo tolerized/BIAP challenged animals developed anaphylactic signs on day 10 and 11, whereafter challenge was stopped. In contrast, BIAP tolerized/BIAP challenged animals did not show any behavioural changes after multiple challenging with BIAP, which was reflected in low BIAP-specific IgG1 titers on day 14 (see Figure 2). In contrast, placebo tolerized/BIAP challenged animals showed high titers of BIAP-specific IgG1 in 2 out of 4 mice of the mid-dose group and 4 out of 4 mice of the high dose group after multiple intravenous dosing with BIAP. In animals of both control groups (placebo tolerized/placebo challenged and BIAP tolerized/placebo challenged), no behavioural changes were observed and no BIAP-specific antibodies were detected.

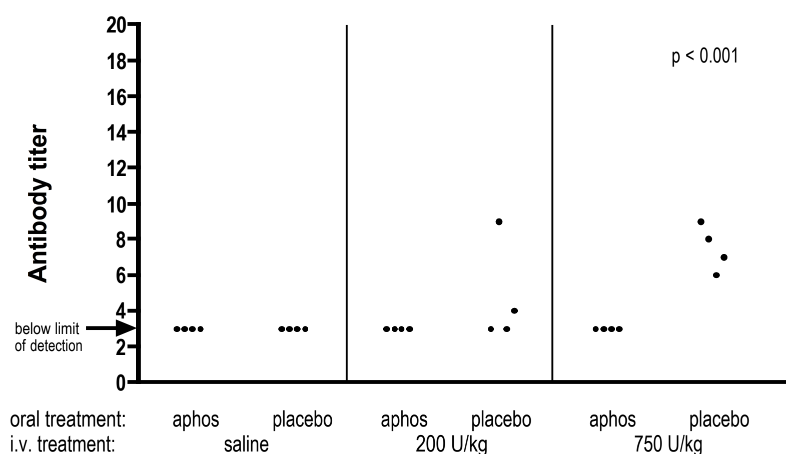

Figure 2: BIAP specific IgG1 titers in serum after 20 days of oral BIAP administration , followed by 11 days of BIAP challenge. Limit of detection equals no measurable IgG1 in the first dilution (2^3 = 1:8) tested. Data of individual mice are depicted. P-values denote statistical significance in IgG1 titers of BIAP-treated groups in comparison to the placebo groups.

Discussion

The medical use of proteins started more than a century ago when immune sera from animal origin were introduced for the prevention or treatment of infections, followed by the use of insulin of bovine and porcine origin some decades later. These products were often immunogenic in patients, sometimes even leading to serious anaphylactic reactions [16]. Initially, the foreign nature of the protein was considered the main cause of immunogenicity. Later on, it was found that products purified from human sera, such as factor VIII, also induced immunologic responses [17]. It is now clear that nearly all biopharmaceuticals derived from both human and non-human sources can induce antibodies [18], although the incidence is much higher in animal-derived than in humanized protein drugs. Many factors such as protein structure, glycosylation, contaminants, formulation, degradation products, route of administration and the dosing regimen influence the immunogenicity of therapeutic proteins [19]. In many cases the antibodies have no or little biological consequences. Problems arise when the antibody neutralizes an endogenous protein with essential biological activity or, even worse, induces anaphylactic shock.

A life-threatening situation occurs when antigen administration induces IgE antibodies. Re-exposure to the antigen will now result in cross-linking of the antigen to IgE molecules, followed by the activation and degranulation of mast cells or basophils within seconds to minutes. This response can cause profound organ dysfunction of the cardiac, respiratory, gastrointestinal, dermal and neural system and is known as anaphylactic shock [20]. Antibodies (including IgM and IgG) can also react with antigen and form immune complexes in the circulation that can be deposited in tissues like the renal glomerulus, skin venules or lungs. Once deposited, immune complexes can trigger the "classical" complement pathway, resulting in the generation of the anaphylatoxins C3a, C4a and C5a which have potent effects on smooth muscle and the vascular system and can also induce degranulation of mast cells and basophils [20]. Complement-containing immune complexes also generate an influx of polymorphonuclear leukocytes into the vessel wall, resulting in inflammation and tissue damage [21].

It has previously been demonstrated that bovine calf intestinal alkaline phosphatase (BIAP) is able to detoxify lipopolysaccharide (LPS) *in vivo* [14]. Recent safety studies with BIAP in various animal species as well as human volunteers showed that single dose BIAP administration to mice, rats, dogs and humans, and multiple-dose BIAP administration to piglets and dogs did not result in any adverse effects, whereas multiple-dosing of BIAP to mice and guinea pigs resulted in anaphylaxis (Beumer *et al.*, in preparation).

In the first part of the present study the safety experiments with mice were repeated in order to study the antibody response after multiple intravenous BIAP dosing. For this purpose, animals were once daily intravenously dosed with BIAP or vehicle for 28 consecutive days. The clinical symptoms observed at day 10 in high dosed mice, but not in low- and mid-dosed animals, combined with time of onset of these symptoms and the presence of BIAP specific IgG1 in day 10 serum samples led us to conclude that animals suffered from anaphylactic shock reactions. Development of anaphylaxis has long been regarded to be mediated by IgE, however, no BIAP-

specific IgE was detected in day 10 serum samples. This was expected as IgE levels normally start to rise 2 weeks after initiation of treatment [22]. Literature suggests that the classical IgE-mediated pathway does not account for all anaphylactic responses. Indeed, Strait *et al.* [23] and Miyajima *et al.* [24] showed that, in contrast to the human situation, antigen-induced anaphylaxis in the mouse primarily proceeds through the IgG1 pathway, even in an experimental model characterized by strong IgE responses.

The main goal of the present study was to investigate if oral treatment with BIAP can prevent anaphylaxis in mice after multiple iv BIAP challenge. To investigate this, mice were orally given BIAP or placebo for 20 consecutive days. After this oral treatment period, mice were once daily intravenously challenged with BIAP or placebo. In contrast to placebo tolerized/BIAP challenged animals, which responded with anaphylactic signs and elevated levels of BIAP-specific IgG1, multiple intravenous BIAP challenge of BIAP tolerized mice did not result in anaphylactic signs or the induction of BIAP-specific IgG1.

In conclusion, this study shows that mice can be orally tolerated to BIAP, thereby preventing the induction of BIAP-specific antibodies and anaphylaxis. Since multiple intravenous BIAP dosing to piglets and dogs did not result in BIAP-specific antibody induction (Beumer *et al.*, in preparation), these species are considered tolerant because of the presence of bovine products in their food. Since humans also consume bovine products in their food, it is expected that also humans tolerate BIAP administration. Indeed, a safety study using human volunteers showed that BIAP-specific antibodies and clinical side-effects were absent. Based on these results, a clinical phase II sepsis trial is currently ongoing.

References

1 Fishman WH. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

2 McComb RB, Bowers GN, Posen S. Alkaline phosphatases. New York: Plenum Press, 1979

3 Narisawa S, Wennberg C, Millan JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. J Pathol 2001; 193:125-133

4 Beckman G, Beckman L, Holm S, et al. Placental alkaline phosphatase types and transplacental IgG transport. Hum Hered 1995; 45:1-5

5 Beckman G, Beckman L, Magnusson SS. Placental alkaline phosphatase phenotypes and pre-natal selection. Evidence from studies of spontaneous and induced abortions. Hum Hered 1972; 22:473-480

6 Beckman L, Beckman G, Mi MP. The relation between human placental alkaline phosphatase types and some perinatal factors. Hum Hered 1969; 19:258-263

7 Beckman L, Bjorling G, Christodoulou C. Pregnancy enzymes and placental polymorphism. I. Alkaline phosphatse. Acta Genet Stat Med 1966; 16:59-73

8 Van Hoof VO, and De Broe. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. crit. rev. clin. lab. sci. 1994; 31:197-293

9 Flock E, Bollman J. Alkaline phosphatase in the intestinal lymph of the rat. J.Biol.Chem. 1948; 175:439-449

10 Mahmood A, Yamagishi F, Eliakim R, et al. A possible role for rat intestinal surfactant-like particles in transepithelial triacylglycerol transport. J Clin Invest 1994; 93:70-80

11 Poelstra K, Bakker WW, Klok PA, et al. A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Invest 1997; 76:319-327

12 Poelstra K, Bakker WW, Klok PA, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169

13 Bentala H, Verweij WR, Huizinga-Van der Vlag A, et al. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566

14 Beumer C, Wulferink M, Raaben W, et al. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. J Pharmacol Exp Ther 2003; 307:737-744

15 Weiner HL. Oral tolerance, an active immunologic process mediated by multiple mechanisms. J Clin Invest 2000; 106:935-937

16 Schernthaner G. Immunogenicity and allergenic potential of animal and human insulins. Diabetes Care 1993; 16:155-165

17 Jacquemin MG, Saint-Remy JM. Factor VIII immunogenicity. Haemophilia 1998; 4:552-557

18 Kromminga A, Schellekens H. Antibodies against erythropoietin and other protein-based therapeutics: an overview. Ann N Y Acad Sci 2005; 1050:257-265

19 Schellekens H. Bioequivalence and the immunogenicity of biopharmaceuticals. Nat Rev Drug Discov 2002; 1:457-462

20 Janeway CA, Travers P, Walport M, et al. IMMUNOBIOLOGY. The immune system in health and disease. 4 ed: Current Biology Publications, 1999

21 Johnson KJ, Chensue SW, Ward PA. Pathology. 3rd ed: Lippincott-Raven Publishers, 1999

22 Von Garnier C, Astori M, Kettner A, et al. In vivo kinetics of the immunoglobulin E response to allergen: bystander effect of coimmunization and relationship with anaphylaxis. Clin Exp Allergy 2002; 32:401-410

23 Strait RT, Morris SC, Yang M, et al. Pathways of anaphylaxis in the mouse. J Allergy Clin Immunol 2002; 109:658-668

24 Miyajima I, Dombrowicz D, Martin TR, et al. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. J Clin Invest 1997; 99:901-914

6

Summary and general discussion

Introduction

Alkaline phosphatases (APs) can be found in many tissues throughout the body where they serve various physiological functions [1-8]. In mammals, this group of enzymes can be classified as either tissue non-specific (TNAP), also called the bone-liver-kidney (BLK) isotype and tissue-specific, which include placental (PLAP), germ cell (GCAP) and intestinal (IAP) isoenzymes [9]. In 1997, it was shown that human placental alkaline phosphatase (HPLAP) dephosphorylates and thereby detoxifies LPS at physiological pH levels [10]. LPS is a constituent of the outer membrane of Gram-negative bacteria and is recognized by host immune cells, thereby generating a systemic immune response responsible for much of the clinical threat associated with Gram-negative sepsis.

Despite all innovations 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 35-50% [11]. Many therapies against this LPS-mediated disease aimed at either neutralizing or antagonizing the production of pro-inflammatory cytokines and mediators or neutralizing the LPS molecule itself [12-17]. However, none of these trials have been proven effective.

Recently, several investigators have shown the promising therapeutic effects of APs. It was observed that HPLAP reduces mortality in mice lethally infected with Gram-negative bacteria; BIAP was shown to attenuate LPS-mediated toxicity in a mouse cecal ligation and puncture model; and inhibition of endogenous intestinal alkaline phosphatase was found to result in prolonged endotoxemia in rats after oral administration of LPS [18-21]. All these observations imply that AP might be beneficial in Gram-negative sepsis [18,19].

This thesis describes the pre-clinical and clinical studies that were initiated in order to investigate if BIAP can be developed as a novel therapeutic agent in the treatment of Gram-negative sepsis. First it was investigated whether BIAP, like HPLAP, exerts the proposed physiological role of LPS detoxification *in vivo* [19]. For this purpose, BIAP was tested in a mouse model in which mice were injected with a lethal dose of *E. coli* bacteria. Mice that did not receive BIAP were used as a control. The results of this study are described in **chapter 2.** The observation that BIAP significantly reduced mortality in mice that had received a lethal dose of *E. coli* bacteria was a clear indication that BIAP may be beneficial in Gram-negative sepsis. However, before BIAP can be applied in the treatment of Gram-negative sepsis, safety and tolerability of its administration had to be investigated. Since it may be required for clinical applications in LPS-overload conditions like sepsis to apply repeated or continuous dosing regimes, single-dose as well as multiple-dose safety studies were performed. **Chapter 4** gives an overview of the single- and multiple-dose safety studies performed in mice, rats and dogs. A multiple-dose safety study using piglets is described in **chapter 2**. The results show that BIAP administration to different animal species resulted in big differences in immune reactivity towards the protein. Single dose administration of BIAP did not result in any adverse effects in mice, rats, dogs and piglets. Multiple-dose administration of BIAP to piglets and dogs did not induce adverse effects as well. In

contrast, mice that had received multiple doses of BIAP reacted with antibody production and serum sickness. The finding that BIAP potentially may give rise to antibody formation and sensitization was further explored in a study with guinea pigs. A possible explanation for the differences in immunological reaction towards BIAP might be the fact that mice do not consume bovine product with their food whereas all the other species tested do.

In order to test the hypothesis that the species differences observed in immune reactivity towards BIAP were due to a lack of tolerance to bovine proteins, mice were orally treated with BIAP for 20 consecutive days, after which mice were immunized by intravenous injection of BIAP. **Chapter 5** shows that long-term oral BIAP administration to mice resulted in tolerization, which was characterized by the absence of clinically relevant toxicity and decreased levels of BIAP-specific antibodies upon intravenous BIAP administration.

Since BIAP administration resulted in big species differences in immune reactivity, safety and tolerability studies had to be performed in man as well. Since humans also consume bovine products (e.g. milk and cheese), no side effects of BIAP treatment are expected. Data obtained in healthy volunteers is summarized in **chapter 4**. Results show that administration of BIAP by continuous infusion does not induce any side effects or antibody production. We therefore concluded that single BIAP administration to humans is safe.

For any therapeutic intervention it is important to know the pharmacokinetic profile. For AP, literature shows that different AP isoforms are cleared from the plasma at various rates [22-24]. The pharmacokinetic behaviour of BIAP was rather unknown and was therefore extensively investigated. Since the cardiovascular physiology of the pig is remarkably similar to that of humans, a piglet model was chosen to study the pharmacokinetics of BIAP. Results of this study are shown in **chapter 2**. In addition, in **chapter 4** the pharmacokinetic profile of BIAP in man was investigated. BIAP clearance showed to be dose-independent and biphasic with an initial half-life of 3-5 minutes (piglets) or 1.68 hours (humans) and a much slower second phase half-life of 2-3 hours (piglets) or 7.9 hours (humans).

The observation that BIAP protected mice against a lethal Gram-negative bacterial insult, most probably by dephosphorylation of the lipid A moiety of LPS, warranted for further study in a LPS-model. For this purpose piglets were used, as they are relatively sensitive to LPS and their cardiovascular physiology closely resembles that of humans. The results of this study are described in **chapter 2**. First, a dose-escalation study was performed in order to determine what LPS dose should be used. Upon this goal, piglets were administered different LPS doses by either intravenous bolus injection or intravenous infusion, after which plasma TNFα levels were determined. Administration of LPS doses of 50 ng per kg bodyweight or higher resulted in detectable TNFα levels. Based upon the TNFα responses as well as the observed changes in hematological and clinical parameters (hearth rate, breathing rate and body temperature), it was concluded that LPS doses of 50 ng/kg could be used in an inflammation model whereas administration of 10 µg/kg more resembled the septic shock situation.

Next, studies were performed in which LPS administration was combined with, or preceded by, BIAP administration. This was done in order to prevent immediate LPS toxicity through interaction with the LPS receptor. In both the sepsis and the inflammation model, BIAP attenuated TNFα production as well as changes in hematological parameters for up to 80%.

Up to date, we and other investigators have shown the very promising therapeutic results for AP. However, little is still known about the mechanism by which AP detoxifies LPS. In order to investigate this mechanism of action and the possible necessary co-factors that facilitate this, an *in vitro* assay was developed, which is described in **chapter 3**. The indirect bioassay most closely resembles the inflamed *in vivo* situation. In this assay, macrophages were incubated with supernatant derived from epithelial cells incubated with LPS with or without BIAP, after which the inflammatory parameters TNFα, IL-6 and NO_x were determined. The results clearly show that BIAP reduced epithelial cell inflammatory responses, which resulted in the reduction of further inflammatory responses from macrophages. Additional studies in this chapter in which epithelial cells and macrophages were pre-incubated with BIAP before LPS exposure, demonstrated that incubation of cells with BIAP results in binding of BIAP to the cells. Based on this observation it was suggested that BIAP needs the cell membrane and/or co-factors associated to it in order to exert its biological effects. Studies in which macrophages were pre-incubated with BIAP showed enhanced detoxification, whereas pre-incubation of epithelial cells with BIAP had no additional effect on the LPS-detoxifying capacity of BIAP. At this moment we do not have an explanation for this remarkable difference. It is a good possibility that it is due to the presence or absence of TLR4 or other molecules involved in LPS signalling on the cell surface but a clear answer can only be obtained by additional research.

It has been demonstrated that GPI-anchored proteins, like APs, easily bind to the cell membrane by means of their GPI-anchor *in vitro* [25,26]. In **chapter 2** we hypothesize that this also happens when BIAP is administrated *in vivo.* In this case, intravenous BIAP administration would result in binding of the protein to the endothelial cells of the blood vessel. This distribution phase is reflected by the fast initial half-life. The much slower second phase clearance is the result of BIAP re-entering the circulation after its dissociation from the endothelial cells.

Thus, administration of BIAP results in binding of BIAP to the cell membrane, a process that is most probably followed by a slow release of BIAP back into the blood or tissues. With this in mind BIAP might not only be used as a therapeutic agent in the treatment of LPS-mediated diseases, but also as a prophylactic therapy at surgeries when the risk of infection with Gram-negative bacteria is high.

Taken together, although the exact mechanism by which BIAP exerts it biological activity remains to be elucidated, BIAP has proven to be effective against LPS-mediated toxicity in different animal models (chapter 2). Although the signs and clinical parameters observed in these models reflect the situation of human sepsis, it is important to evaluate the clinical relevance of animal models.

Animal models of sepsis compared to human sepsis – clinical relevance

Animal models provide insight in specific components of the septic process but do not really mimic the full clinical complexity and heterogeneity of patients with sepsis. Because they provide fundamental information about pharmacokinetics, toxicity and mechanisms of drug interaction that cannot be duplicated by other methods, animal models will remain essential in the development of all new therapies for sepsis and septic shock. The ideal animal model is simple and reproducible. The animals should show signs of sepsis, including cytokine and hematological responses and the illness should progress from a localized infection through systemic infection to multiple organ failure [27]. Several animal models can be used to induce Gram-negative sepsis. They include intravascular or intraperitoneal administration of viable bacteria, infusion of LPS, cecal ligation and puncture, soft tissue infections, pneumonia models and meningitis models [27-29]. Many different animal species have been used, including mice, rats, rabbits, dogs, piglets, sheep and non-human primates [29]. Although in these models the signs and clinical parameters observed in human sepsis are reflected, it is important to evaluate the extent to which they mimic the septic response [30].

Infusion of large doses of viable bacteria results in reproducible but dramatic physiological responses. Although this model mimics situations of extreme clinical sepsis (for example meningococcemia), this is not likely to happen in the clinical situation. Despite this, models that use infusion of viable bacteria to induce sepsis in animals can still be used when certain limitations are recognized. For instance, most patients are not challenged with a lethal bacterial load but rather harbour a septic focus that showers the body with bacteria. It is also known that bacteria infused in high doses do not colonize nor replicate *in vivo* [27].

Infusion of LPS mimics many of the features associated with Gram-negative sepsis [29] but LPS sensitivity varies between species [31,32]. For instance, small mammals and baboons are relatively insensitive to the effects of LPS, whereas rabbits, sheep and chimpanzees show an enhanced response [33]. Despite the observation that lower LPS doses represent more physiological conditions, especially in their resemblance with human sepsis and its association with coagulopathy and multi-organ dysfunction, most LPS studies have continued to use high LPS doses in non-sensitized animals. Since (killed) bacteria are much more lethal than LPS alone, caution is needed when assessing the clinical efficacy of novel therapeutic agents in animals models of endotoxemia. Nowadays, it is generally accepted that LPS injection may serve as a model for endotoxic shock but not for sepsis [29,34].

Other methods to induce sepsis include breaking the natural defence of the gastrointestinal tract to allow endogenous bacteria to establish an infection, and the generation of abscesses in soft tissues of the limbs [35]. The advantage of these models is that it results in contamination with mixed bacterial flora in the presence of hampered tissues, thereby closely resembling clinical problems [27].

Conclusions

Although BIAP is cleared much faster than HPLAP, it attenuates LPS-mediated effects in mice and piglets for up to 80%. In addition, safety studies performed in different animal species showed that single dose administration of BIAP up to 5354 U/kg bodyweight is safe. Humans are not expected to show BIAP-related side effects upon administration of BIAP since they consume bovine products with their food. Based on efficacy of the protein as well as safety and tolerability after its administration, BIAP potentially encompasses a novel therapeutic drug in the treatment of LPS-mediated diseases. Results of a clinical phase II in sepsis patients, which is currently ongoing, will learn us if BIAP is indeed beneficial in Gram-negative sepsis.

Future perspectives

Despite the promising therapeutic results obtained with BIAP, little is still known about the mechanisms by which AP detoxifies LPS. In chapter 3 of this thesis a bioassay is described in which these mechanisms can be studied more closely, for example on HEK293T cells. It is also possible to use HEK293T cells transfected with TLR4, CD14, and MD2. In order to investigate which molecules of the LPS signalling cascade are involved in the detoxification process, one or more of these molecules can be blocked. Another way to study the working mechanism of AP in more detail is to use labelled LPS and/or AP. In this way it is possible to study the interaction between LPS and AP more closely.

When BIAP is going to be used in the treatment of LPS-mediated diseases, it will most probably be applied intravenously. Koyama *et al.* [21] already reported that AP-treated endothelial cells were more viable after LPS administration than cells treated with LPS alone. It would be of great interest to study if BIAP can also reduce LPS-mediated endothelial cell responses, like production of the cytokines IL-6 and IL-8 and the expression of tissue factor and the adhesion molecules VCAM, ICAM and E-selectin. In addition, co-cultures can be performed in which the effect of AP on the interaction between neutrophils and endothelial cells can be studied.

The above-suggested experiments will provide more data on the relevance of BIAP in the innate immune system. A better understanding of the mechanisms of action of BIAP might result in a more effective treatment of patients suffering from LPS-mediated diseases. It is not expected that a complicated disease like Gram-negative sepsis can be cured by one drug. However, a drug that detoxifies one of the major initiators of this disease might well be included in a therapeutic combination with other drugs, like Activated Protein C.

Besides the possible application of BIAP in the treatment of Gram-negative sepsis, BIAP might also be beneficial in other diseases. In contrast to Gram-negative sepsis, which is a relatively acute disease, colitis is a disease in which inflammation is chronic. It is now known that LPS, at least in part, mediates this inflammation. Since BIAP has been shown to effectively reduce acute

LPS-mediated effects, it would be of great interest to explore the therapeutic possibilities of BIAP intervention in chronic LPS-mediated diseases.

Given the short $t_{1/2}$ of BIAP, it may be required for clinical application to apply repeated or continuous dosing regimes in LPS-overload conditions. Safety studies with BIAP showed that administration of BIAP according to a multiple-dosing regimen might result in antibody formation, a situation that is not likely to occur in humans but at least care should be taken. Since administration of a human-derived protein is less likely to induce immunogenicity, a human-derived AP might be a good and safe alternative. An AP isoenzyme that may be suitable for this is HPLAP. Since this is a human-derived protein, it is not expected to be immunogenic in man. It also has a half-life time of several days, which most certainly makes it an interesting alternative for BIAP. Since large amounts of placental-derived HPLAP are difficult to get, recombinant HPLAP seems to be the ideal therapeutic.

References

1 Narisawa S, Wennberg C, Millan JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. J Pathol 2001; 193:125-133

2 Beckman G, Beckman L, Holm S, et al. Placental alkaline phosphatase types and transplacental IgG transport. Hum Hered 1995; 45:1-5

3 Beckman G, Beckman L, Magnusson SS. Placental alkaline phosphatase phenotypes and pre-natal selection. Evidence from studies of spontaneous and induced abortions. Hum Hered 1972; 22:473-480

4 Beckman L, Beckman G, Mi MP. The relation between human placental alkaline phosphatase types and some perinatal factors. Hum Hered 1969; 19:258-263

5 Beckman L, Bjorling G, Christodoulou C. Pregnancy enzymes and placental polymorphism. I. Alkaline phosphatse. Acta Genet Stat Med 1966; 16:59-73

6 Van Hoof VO, and De Broe. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. crit. rev. clin. lab. sci. 1994; 31:197-293

7 Flock E, Bollman J. Alkaline phosphatase in the intestinal lymph of the rat. J.Biol.Chem. 1948; 175:439-449

8 Mahmood A, Yamagishi F, Eliakim R, et al. A possible role for rat intestinal surfactant-like particles in transepithelial triacylglycerol transport. J Clin Invest 1994; 93:70-80

9 Fishman WH. Alkaline phosphatase isozymes: recent progress. Clin Biochem 1990; 23:99-104

10 Poelstra K, Bakker WW, Klok PA, et al. A physiologic function for alkaline phosphatase: endotoxin detoxification. Lab Invest 1997; 76:319-327

11 Vincent JL, Abraham E, Annane D, et al. Reducing mortality in sepsis: new directions. Crit Care 2002; 6 Suppl 3:S1-18
12 Riedemann NC, Ward PA. Anti-inflammatory strategies for the treatment of sepsis. Expert Opin Biol Ther 2003; 3:339-350
13 Ghiselli R, Giacometti A, Cirioni O, et al. Neutralization of endotoxin in vitro and in vivo by Bac7(1-35), a proline-rich antibacterial peptide. Shock 2003; 19:577-581
14 Lynn WA. Anti-endotoxin therapeutic options for the treatment of sepsis. J Antimicrob Chemother 1998; 41 Suppl A:71-80
15 Pollack M, Ohl CA, Golenbock DT, et al. Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induced proinflammatory functions. J Immunol 1997; 159:3519-3530
16 Reinhart K, Karzai W. Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned. Crit Care Med 2001; 29:S121-125
17 von der Mohlen MA, Kimmings AN, Wedel NI, et al. Inhibition of endotoxin-induced cytokine release and neutrophil activation in humans by use of recombinant bactericidal/permeability-increasing protein. J Infect Dis 1995; 172:144-151
18 Poelstra K, Bakker WW, Klok PA, et al. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. Am J Pathol 1997; 151:1163-1169
19 Bentala H, Verweij WR, Huizinga-Van der Vlag A, et al. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. Shock 2002; 18:561-566
20 van Veen SQ, van Vliet AK, Wulferink M, et al. Bovine intestinal alkaline phosphatase attenuates the inflammatory response in secondary peritonitis in mice. Infect Immun 2005; 73:4309-4314
21 Koyama I, Matsunaga T, Harada T, et al. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. Clin Biochem 2002; 35:455-461
22 Blom E, Ali MM, Mortensen B, et al. Elimination of alkaline phosphatases from circulation by the galactose receptor. Different isoforms are cleared at various rates. Clin Chim Acta 1998; 270:125-137
23 Hoffmann WE, Dorner JL. Disappearance rates of intravenously injected canine alkaline phosphatase isoenzymes. Am J Vet Res 1977; 38:1553-1556
24 Hoffmann WE, Renegar WE, Dorner JL. Serum half-life of intravenously injected intestinal and hepatic alkaline phosphatase isoenzymes in the cat. Am J Vet Res 1977; 38:1637-1639
25 Low MG, Zilversmit DB. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918
26 Medof ME, Nagarajan S, Tykocinski ML. Cell-surface engineering with GPI-anchored proteins. Faseb J 1996; 10:574-586
27 Parker SJ, Watkins PE. Experimental models of gram-negative sepsis. Br J Surg 2001; 88:22-30
28 Garrido AG. Experimental models of sepsis and septic shock: an overview. Acta Cir. Bras. 2004; 19
29 Fink MP, Heard SO. Laboratory models of sepsis and septic shock. J Surg Res 1990; 49:186-196
30 Piper RD, Cook DJ, Bone RC, et al. Introducing Critical Appraisal to studies of animal models investigating novel therapies in sepsis. Crit Care Med 1996; 24:2059-2070
31 Redl H, Bahrami S, Schlag G, et al. Clinical detection of LPS and animal models of endotoxemia. Immunobiology 1993; 187:330-345

32 Moore FD, Jr., Moss NA, Revhaug A, et al. A single dose of endotoxin activates neutrophils without activating complement. Surgery 1987; 102:200-205

33 McCuskey RS, McCuskey PA, Urbaschek R, et al. Species differences in Kupffer cells and endotoxin sensitivity. Infect Immun 1984; 45:278-280

34 Riedemann NC, Guo RF, Ward PA. The enigma of sepsis. J Clin Invest 2003; 112:460-467

35 Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock--a review of laboratory models and a proposal. J Surg Res 1980; 29:189-201

7

Nederlandse samenvatting

Introductie

In een gezonde situatie zijn er altijd bacteriën in of op de mens aanwezig als onderdeel van de normale bacteriële flora van huid, slijmvliezen en darmen (commensale microflora). Echter, explosieve vermeerdering of verspreiding van deze commensalen of van indringers van buitenaf door de lichaamsweefsels kan leiden tot infectie. De binnengedrongen micro-organismen produceren toxinen (zoals bijvoorbeeld lipopolysaccharide; LPS) die in het bloed kunnen komen en zo een ontstekingsreactie veroorzaken.

LPS is een molecuul, dat zich aan en deels in de buitenwand van bepaalde bacteriën (Gram-negatieve bacteriën) bevindt en is belangrijk voor de levensvatbaarheid van de bacterie. Als de bacterie zich deelt of wordt vernietigd door bijvoorbeeld antibiotica, komt dit LPS vrij. Het kan dan aan allerlei eiwitten en andere moleculen van de gastheer binden en zo het immuunsyssteem activeren. Deze reactie, waarbij bepaalde cellen van het immuunsysteem zijn betrokken, zorgt er in de meeste gevallen voor dat de patiënt het met zo weinig mogelijk schade overleeft. Helaas komt het voor dat de immuunrespons niet in balans is met de schade die de indringer heeft veroorzaakt. Zo'n doorgeslagen respons wordt ook wel sepsis genoemd. Sepsis kan het gevolg zijn van verwonding, verbranding, nier- of galstenen en darmaandoeningen. Ook ernstige ziekten zoals aangeboren en verworven defecten van het immuunsysteem, kanker, levercirrose, slechte nierwerking en diabetes mellitus kunnen sepsis veroorzaken. Risicovol zijn ook ingrijpende medisch-technische handelingen waardoor de natuurlijke barrière van het lichaam wordt doorbroken, zoals chirurgische ingrepen en het plaatsen van katheters in bloedvaten, prothesen, urinekatheters en beademingstubes. Ook medicijnen, zoals cytostatica, corticosteroïden en soms antibiotica kunnen een rol spelen.

Patiënten met sepsis zijn acuut en ernstig ziek en moeten in het ziekenhuis worden opgenomen en behandeld. Doordat in een tijdsbestek van uren tot dagen het falen van organen als nieren, lever en longen kan optreden is de kans groot dat de patient overlijdt als er niet snel wordt gehandeld. De meeste gevallen van sepsis worden veroorzaakt door bacteriën, maar ook virussen, parasieten, gisten of schimmels kunnen sepsis veroorzaken. Door bacteriën veroorzaakte sepsis, met in het bijzonder door Gram-negatieve bacteriën veroorzaakte sepsis, is wereldwijd nog altijd een groot klinisch probleem. Het aantal neemt nog steeds toe en met de toename in chronische ziekten, het gebruik van immuunonderdrukkende medicatie, zware ingrepen, resistente micro-organismen en steeds ouder wordende mensen wordt een verdere toename verwacht. Tegelijkertijd is er nog steeds geen therapeutische behandeling voor de immunologische onbalans die verantwoordelijk is voor deze ziekte.

Gedurende de laatste twee decennia is er veel onderzoek gedaan naar nieuwe manieren om sepsis te behandelen. Hierbij richtte men zich vooral op het neutraliseren van de verschillende ontstekingsmediatoren die tijdens sepsis worden geproduceerd. Dit bleek vaak goed te werken in diermodellen, maar in de mens kon het effect vaak niet worden aangetoond. In werkelijkheid zijn de ontstekingsmediatoren die tijdens sepsis worden aangemaakt nodig om de infectie te

bestrijden. Het remmen of wegvangen van deze mediatoren kan daarom schadelijk zijn voor de patiënt. Daarbij komt dat het remmen van "slechts" één van de mediatoren niet veel effect heeft omdat er nog vele andere mediatoren, en hun effecten, aanwezig zijn. De beste manier om sepsis te behandelen lijkt daarom een methode die ingrijpt op een punt vooraan in de sepsis respons bij (een van) de veroorzaker(s): het LPS-molecuul.

Methoden waarin het LPS molecuul werd geneutraliseerd door middel van antistoffen of LPS-bindende eiwitten leken aanvankelijk te werken omdat ze de immuunrespons maskeerden. Maar omdat de sepsis veroorzakende bacterie nog steeds aanwezig was kon deze - nu het immuunsysteem omzeild was - vrij zijn gang gaan. Naast onderzoek naar nieuwe behandelmethoden werd er ook veel onderzoek gedaan naar LPS. Een van de vindingen die werd gedaan was dat als je het LPS molecuul defosforyleert, dat wil zeggen dat je er een fosfaatgroep afhaalt, het overgebleven molecuul niet of nauwelijks meer in staat was een immuunrespons op gang te brengen. Ondank deze kennis werd tot eind jaren 90 nooit een poging ondernomen om LPS enzymatisch te ontdoen van een fosfaatgroep.

In 1997 werd aangetoond dat het enzym alkalisch fosfatase (ALF) afkomstig uit de placenta van de mens LPS defosforyleert en daarmee onschadelijk maakt (detoxificeert). Later werd aangetoond dat dit enzym sterfte voorkwam bij muizen die met een dodelijke hoeveelheid Gram-negatieve bacteriën waren ingespoten. Aangezien ALF in het lichaam op verschillende plaatsen voorkomt waar je liever geen infecties hebt, zoals in de darmen, placenta, lever en nieren, werd gesuggereerd dat het weleens een belangrijke rol kon spelen in de bescherming tegen Gram-negatieve sepsis. Echter, voordat ALF kan worden toegepast in de behandeling van Gram-negatieve sepsis moeten er nog aanvullende pre-klinische en klinische studies worden gedaan. Deze studies, die uitgebreid worden beschreven in dit proefschrift, waren zo ontworpen dat de resultaten ervan een goed inzicht geven in de werking en de veiligheid van ALF, hetgeen een goede bijdrage levert aan zijn ontwikkeling tot een nieuw medicijn voor de behandeling van LPS-gemedieerde ziekten.

In **hoofdstuk 1** wordt een algemeen overzicht gegeven van LPS. Er wordt behandeld wat LPS voor molecuul is, hoe het lichaam reageert als LPS door cellen van het immuunsysteem wordt waargenomen en hoe dit kan kan leiden tot ziekten zoals Gram-negatieve sepsis. Verder wordt dieper ingegaan op het hoe en wat van Gram-negatieve sepsis en wat er allemaal al geprobeerd is in de strijd een geneesmiddel te ontwikkelen tegen deze ziekte. Tenslotte wordt het lichaamseigen enzym alkalisch fosfatase (ALF) aangedragen als een mogelijk nieuw medicijn. Dit promotieonderzoek werd gedaan met als hoofddoel dit enzym verder te kunnen ontwikkelen als nieuw geneesmiddel in de bestrijding van LPS-gemedieerde ziekten.

Zoals reeds in de inleiding genoemd werd in 1997 aangetoond dat humaan placentair alkalisch fosfatase (HPLAP) in staat is om LPS te defosforyleren en daarmee te detoxificeren. Op basis van deze resultaten werd gezegd dat dit enzym wel eens een nieuw geneesmiddel kon zijn voor de behandeling van LPS-gemedieerde ziekten. Omdat er op snelle manier zeer veel ALF kan worden geïsoleerd uit kalverdarmen (bovine intestinaal alkalisch fosfatase; BIAP), werd voor de

studies beschreven in dit proefschrift geen gebruik gemaakt van HPLAP maar van BIAP. Of dit BIAP, net als HPLAP, in staat was om *in vivo* (in het dier) LPS onschadelijk te maken, werd in **hoofdstuk 2** onderzocht. Om dit te testen kregen muizen een dodelijke hoeveelheid bacteriën in de buikholte ingespoten. 80% van de dieren die naast bacteriën ook BIAP hadden gekregen, overleefde deze infectie. Van de dieren die geen BIAP hadden gekregen overleefde slechts 20%. Om de LPS-ontgiftende werking van BIAP verder te testen, werd getest in een varkensmodel. Hiervoor kregen jonge varkens LPS toegediend in een dosis tussen de 10 nanogram en 10 microgram per kilo lichaamsgewicht, al dan niet voorafgegaan door BIAP. Hierna werd op vaste tijdstippen bloed afgenomen dat werd gescreened op verschillende bloedwaarden en op het ontstekingseiwit TNFα. Uit deze studies kwam naar voren dat behandeling met BIAP de door LPS-geïnduceerde TNFα levels verlaagt. Ook veranderingen in celsamenstelling van het bloed werden door BIAP verminderd. Naast de studies waarin werd gekeken naar de werking van BIAP, beschrijft dit hoofdstuk ook het farmacokinetisch profiel (wat het lichaam met de stof doet) van BIAP en de veiligheid van 28 dagen intraveneus (rechtstreeks in de bloedbaan) toedienen bij het varken. De resultaten van de kinetiek experimenten laten zien dat BIAP in twee fasen uit het bloed wordt verwijderd met een snelheid van enkele minuten tot enkele uren. De resultaten van de veiligheidsstudies laten zien dat 28 dagen intraveneus toedienen van BIAP veilig is in het varken. Om echter een goede uitspraak te kunnen doen over de veiligheid is het verplicht dat een mogelijk nieuw geneesmiddel in meerdere grote en kleine diersoorten wordt getest.

In **hoofdstuk 3** laten we zien dat BIAP ook *in vitro* (in een cellijn) LPS kan detoxificeren. Om dit aan te tonen werden macrofagen en epitheelcellen geïncubeerd met LPS, met of zonder BIAP. Macrofagen zou je kunnen vergelijken met een soort stofzuigers, ze ruimen alles op wat niet in het lichaam hoort. Epitheelcellen zijn de cellen die onze organen aan de buitenkant bekleden. Zowel macrofagen als epitheelcellen behoren tot de cellen die als een van de eersten in aanraking komen met schadelijke invloeden van buitenaf, zoals bacteriën en hun producten. De resultaten van dit hoofdstuk laten zien dat BIAP in staat is om de door macrofagen geproduceerde cytokinen (signaal eiwitten) als TNFα en IL-6, alsmede het door epitheelcellen geproduceerde IL-8 te remmen. Ook tonen we hier aan dat voor een goede werking BIAP eerst aan de cel moet binden.

Hoofdstuk 4 behandelt de veiligheidsstudies met BIAP in verschillende diersoorten. In eerste instantie werden muizen en honden een- of meermalig ingespoten met BIAP. Omdat deze twee diersoorten zo verschillend reageerden werden meer diersoorten getest. Naast de veiligheid van BIAP in verschillende diersoorten werd ook de veiligheid van toediening van BIAP aan de mens bestudeerd. Ook werd gekeken naar het farmacokinetisch gedrag in de mens. De resultaten van deze studies laten zien dat eenmalige toediening aan muizen, ratten, honden, varkens en ook aan mensen geen bijverschijnselen tot gevolg heeft. Het meermalig toedienen van BIAP aan muizen en cavia's, daarentegen, resulteert in antilichaamvorming en toxische effecten, terwijl meermalige toediening aan varken, hond en mens geen neveneffecten heeft. Overeenkomstig met de resultaten van de kinetiek experimenten in het varken wordt BIAP bij de mens ook in twee fasen uit het bloed verwijderd, zij het met een snelheid van enkele uren.

In het laatste experimentele hoofdstuk, **hoofdstuk 5,** wordt er verder ingegaan op de, in hoofdstuk 4 gevonden, toxiciteit (neveneffecten) van meermalige toediening van BIAP aan muizen en cavia's. Omdat alleen deze dieren last kregen van bijverschijnselen en varkens, honden en de mens niet, word de hypothese geopperd dat laatst genoemden misschien tolerant zijn doordat ze door middel van hun eten zijn blootgesteld aan eiwit afkomstig van koeien. De resultaten van hoofdstuk 3 worden hier gereproduceerd en er wordt aangetoond dat de in muis en cavia geobserveerde neveneffecten immunologisch-gemedieerd zijn. Tevens laten we in dit hoofdstuk zien dat deze immunotoxiciteit kan worden voorkomen door muizen gedurende 28 dagen BIAP toe te dienen in hun drinkwater en ze zo tolerant te maken voor BIAP.

In **hoofdstuk 6** worden de belangrijkste resultaten samengevat en bediscussieerd. De eindconclusie is dat BIAP, gezien de goede LPS-detoxificerende capaciteiten *in vitro* en *in vivo* en de afwezigheid van toxiciteit in de mens, een zeer sterke kandidaat is om verder te worden ontwikkeld als nieuw geneesmiddel voor de behandeling van LPS-gemedieerde ziekten. Op dit moment is er een studie gaande waarin de effectiviteit van BIAP wordt getest in sepsispatienten.

List of abbreviations

γ-GT/ GGT	gamma- glutamyltransferase
ALAT	alanine aminotransferase
ALP / APs	alkaline phosphatase(s)
ANOVA	analysis of variance
BUN	blood urea nitrogen
APC	activated protein C
ARDS	acute respiratory distress syndrome
ASA	active systemic anaphylaxis
ASAT	aspartate aminotransferase
ASGPR	asialoglycoproteinreceptor
ATCC	American tissue culture collection
AT-III	antithrombin-III
AUC	area under the curve
BHI medium	brain heart infusion-medium
BIAP	bovine calf intestinal alkaline phosphatase
BLK	bone-liver-kidney
BPI	bactericidal permeability-increasing protein
bpm	beats per minute
BSA	bovine serum albumine
bw	body weight
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
Chol	cholesterol
Cl	clearance
Cmax	maximum concentration
DC	dendritic cell
DIC	disseminated intravascular coagulation
ECG	electrocardiogram
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanate
GCAP	germ cell alkaline phosphatase
GDL	gemeenschappelijk dieren laboratorium
GLP	good laboratory practice
GMP	good manufacturer practice
GPI	glycosylphosphatidylinositol
HDL	high density lipid
HE	hematoxilin-eosin
HPLAP	human placental alkaline phosphatase
i.d.	intradermal
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IAP	intestinal alkaline phosphatase
ICAM-1	intercellular adhesion molecule-1
ICU	intensive care unit
IE	international unit

IFNγ	interferron-gamma
Ig	immunoglobulin
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
LB medium	Luria Bertani medium
LBP	LPS binding protein
LDH	lactatedehydrogenase
LDL	low density lipid
LPS	lipopolysaccharide
mCD14	membrane-bound CD14
MCP-1	macrophage chemoattracktant protein-1
MIP-1	macrophage inhibitory protein-1
MOF	multi-organ failure
MPLA	monophosphoryl lipid A
MPLPS	monophosphoryl lipopolysaccharide
MSR	macrophage scavenger receptor
NFκB	nuclear factor-kappa B
NO	nitric oxide
OD	optical density
OECD	organization for economic cooperation and development
OG	octylglucoside
OVA	ovalbumine
PAF	platelet activating factor
PAS	periodic acid schiff
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCA	passive cutaneous anaphylaxis
PE	phycoerythrin
Pi	anorganic phosphate
PLAP	placental alkaline phosphatase
PMN	polymorphonuclear granulocyte
pNPP	paranitrophenylphosphate
RPE-Cy5	phycoerythrin-Cy5
rpm	rotations per minute
S.E.M.	standard error of the mean
sCD14	soluble CD14
SD	standard deviation
SIRS	systemic inflammatory response syndrome
sTNFR	soluble tumour necrosis factor receptor
$t_{1/2}$	half-life time
TBS	tris buffered saline
TBST	tris-buffered saline with 0.05% tween-20
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	toll-like receptor
TNAP	tissue non-specific alkaline phosphatase
TNFα	tumour necrosis factor-alpha
TNFR	tumour necrosis factor receptor
U	unit(s)
UC	ulcerative colitis
v/v	volume over volume
VCAM-1	vascular cell adhesion molecule-1
Vd	volume of distribution
w/v	weight over volume
WBC	white blood cell

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Dankwoord

Raar is dat.....
Na een periode heel erg druk geweest te zijn met schrijven en layouten is het moment nu daar: mijn manuscript is af! Ik doe mijn labtop dicht. Eerst niets, dan bekruipt me het onbehaaglijke gevoel dat ik niet moet zitten niksen maar wat moet gaan doen, maar wat...? Tijd genoeg nu dus om terug te kijken op de afgelopen 4 jaar en iedereen te bedanken die op zijn of haar manier een bijdrage heeft geleverd aan dit proefschrift. (Wat is er veel gebeurd).

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Curriculum Vitae

Géneviève Chantal Beumer werd geboren in Utrecht op 30 januari 1976. In 1994 behaalde zij haar havo diploma aan het Koningin Wilhelmina College in Culemborg. In datzelfde jaar begon zij aan haar studie proefdierkunde (Hoger Laboratorium Onderwijs) aan de Hogeschool van Utrecht. In haar laatste jaar liep zij stage bij de afdeling experimentele radiotherapie van het AMC in Amsterdam. Zij deed hier onderzoek naar een combinatie van chemotherapie en hyperthermie als mogelijk nieuwe behandelmethode voor kankerpatienten. Na haar afstuderen in 1998 begon zij haar (verkorte) studie Biologie aan de Universiteit van Utrecht. Tijdens de doctoraalfase liep zij stage bij de groep immunotoxicologie van het Institute for Risk Assessment Sciences (IRAS) van de Universiteit van Utrecht waar zij werd begeleid door Drs. Colin de Haar en Dr. Raymond Pieters. Aansluitend op het behalen van haar doctoraal diploma in 2001 begon zij als AIO aan het onderzoek beschreven in dit proefschrift. Dit onderzoek werd uitgevoerd bij het IRAS in samenwerking met AM-Pharma Holding BV te Bunnik onder begeleiding van prof. dr. Willem Seinen.