Full Length Research Paper

Effects of CpG motifs on mortality and lung inflammation in a rat model of endotoxemia

Stephan Dueren*[1], Koroush Kabir*[1], Lewis F. Neville[2], Eva Steinringer[1], Guido Grass[3], Micaela Mathiak[4], Peter Behrens[4], Christoph Rangger[1], Lutz Besch[6], Thomas Minor[5], and Guenther Mathiak[6*]

[1]Department of Trauma Surgery, University Hospital of Bonn Medical School, Bonn, Germany
[2]TransPharma Medical Ltd., Lod, Israel
[3]Coordinating Center for Clinical Trials, University of Cologne, Cologne, Germany
[4]Institute of Pathology, University Hospital of Bonn Medical School, Bonn, Germany
[5]Experimental Division, Department of Surgery, University Hospital of Bonn Medical School, Bonn, Germany
[6]Department of Trauma Surgery, University Hospital of Kiel Medical School, Kiel, Germany.
*Both authors contributed equally to the manuscript.

Pathogen associated microbial patterns (PAMP) such as lipopolysaccharides (LPS) and CpG-oligodeoxynucleotides (CpG-ODN) are recognized by innate immunity via pattern recognition receptors (PRR), e.g. Toll-like receptors (TLR). Most of them involve recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) followed by initiation of the ongoing signaling pathway. Preliminary studies demonstrated that CpG-ODN application induces cross-tolerance to LPS treatment *in vitro*. The present study investigated whether ODN containing CpG motifs could induce hyporesponsiveness following LPS treatment and reduce mortality. Interleukin (IL)-6 was determined in bronchoalveolar lavage (BAL) fluid as well lung histopathological changes. In total, 188 male Sprague-Dawley rats were randomly assigned to the following groups: In a first series, mortality was observed. To that end, animals received CpG-ODN or control-ODN i.p. (12 or 48 nmol) 8 hours, respectively 7 days prior to the bolus administration of i.p. endotoxin (25 mg/kg (=LD_{80}) E. coli 0111:B4 LPS). Animals were monitored 7 days for mortality. In a second series, rats were treated with 12 nmol CpG-ODN or control-ODN i.p. 8 hours, respectively 7 days prior to the i.p. bolus administration of endotoxin (10 mg/kg (=LD_0)). 3, 12 and 36 hours after LPS administration blood samples were drawn for blood cell counts. Organs were harvested, weighed and examined histopathologically. The BAL IL-6 levels were determined by ELISA. Mortality in the CpG-ODN treated groups was not significantly different from control-ODN groups. Blood cell counts, lung wet weight and histopathology of different organs did not show any different effects between CpG-ODN and control-ODN. No significant differences in BAL IL-6 levels between the groups could be demonstrated. In conclusion, positive *in vitro* data could not be transferred to a rat model of endotoxemia.

Keywords: CpG, oligodeoxynucleotide, lipopolysaccharide, rat model, endotoxemia, lung injury.

INTRODUCTION

Despite several improvements in sepsis therapy, systemic inflammatory response syndrome (SIRS), sepsis and septic shock are still the major cause of death on ICU worldwide. Improvement in mortality of septic patients could only be achieved by supportive therapy on ICU, e.g. advanced ventilatory regimen in lung injury,

hemofiltration in renal failure. Unfortunately, none of the promising results apart from the PROWESS study (Bernard et al., 2001), could be transferred from the bench to the bedside. The results of the PROWESS were minimized by the ADDRESS study (Abraham et al., 2006).

To date, no curative therapy targeting the initiated overwhelming mediator cascade is available so far. Symptomatic treatment in most of the cases starts in a running progress of different mediators and cascades

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*Corresponding author E-mail: guenthermathiak@yahoo.de

with already existing damages. Nevertheless, during the last decade major novel insights into signaling pathways of sepsis were achieved.

Trigger for a pathogen-specific answer of the innate immunity are pathogen associated microbial patterns (PAMP). These PAMPs are recognized by the cells of the innate immunity (granulocytes, dendritic cells (DC), monocytes, natural killer (NK)-cells) via pattern recognition receptors (PRR). An important group of PRRs are the Toll-like receptors (TLR). To date, thirteen TLRs and their ligands have been identified (Tabeta et al., 2004; Akira and Takeda, 2004). The most effective PAMP is lipopolysaccharide (LPS), recognized by TLR-4, releasing different proinflammatory cytokines, e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-10. Other PAMPs are synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides (CpG motifs), which are more often part of the bacterial than the DNA of vertebrates. They are recognized by TLR-9 and activate plasmocytoid DC, macrophages and B-cells. In contrast to TLR-4, TLR-9 is expressed in endosomal compartments and not on the surface of immune competent cells (Klinmann, 2004).

Even in high doses CpG-ODN is well tolerated in animal studies including primates (Jones et al., 1999) and leads to a highly selected activation of the innate immunity.

The development of LPS-tolerance after repetitive application is well known *in vivo* and *in vitro*. Subsequent stimulation of TLR-2, -4, -5 and -9 with their own ligands leads to hyporesponsiveness (Dalpke et al., 2005).

Most of the TLRs, e.g. TLR-4 and TLR-9 are recruiting adaptor molecule myeloid differentiation factor 88 (MyD88) followed by initiation of the ongoing signaling pathway. Therefore, cross-tolerances are highly probable. Indeed, cross-tolerances between TLR-2 and -4, as well as TLR-4 and -9 are reported in the literature ((Sato et al., 2000; Yeo, 2003). In addition, CpG-ODN and LPS have been shown to act synergistically and lead to a massive proinflammatory cytokine release (Yi et al., 2001).

Up to date, it is unknown whether pretreatment with CpG-ODN is capable to induce tolerances following endotoxemia with a significant reduction of mortality and downstream of the inflammatory reaction in a rat model of endotoxemia.

MATERIALS AND METHODS

Chemicals and reagents

Phosphorothioate modified ODN was custom synthesized and purified by MWG Biotec AG (Munich, Germany). The sequence of the CpG-ODN was 5´-TTCATGACGTTCCTGATGCT-3´ (Weighardt et al., 2000) and contains one of the most stimulatory CpG-motifs: GACGTT (Krieg et al., 1995). The sequence of the control ODN (5´-GCTTGATGACTCAGCCGGAA-3´) does not contain effective CpG motifs. The control ODN was chosen, because it did not show any

biological activity in various experimental systems tested (Weighardt et al., 2000). Endotoxin was purchased as lyophilized, purified Escherichia coli 0111:B4 LPS from Sigma Aldrich (Munich, Germany). Ketamine (Ketavet®, 100 mg/ml) was supplied by Pharmacia and Upjohn GmbH (Erlangen, Germany) and Xylazine (Rompun®, 20 mg/ml) was supplied by Bayer AG (Leverkusen, Germany).

Animals

All experiments were performed using male Sprague-Dawley rats (Harlan Winkelmann, Borchen, Germany) of 300 ± 50 g body weight on the day of ODN treatment. Animals were housed under standardized conditions in single cages with free access to diet and water. All experiments conformed to the Deutsches Tierschutzgesetz and NIH guidelines for animal research. Animal protocol approval was authorized by the animal committee (Municipality of Bonn).

Preliminary mortality study

47 rats were injected intraperitoneally (i.p) in different dosages per kilogram body weight of LPS to define a LD_{80} as well as survivors in control groups.

Experimental protocol

Anesthesia was initiated by intramuscular injection of a ketamine/ xylazine solution containing 90 mg ketamin/ kg and 2 mg xylazine/ kg body weight.

In total, 188 rats were randomly assigned to the following groups: In a first series mortality was observed: To that end, 116 animals in 8 groups (Table 1) received CpG-ODN or control-ODN i.p. at a dosage of 12, respectively 48 nmol. 8 hours or 7 days after administration of ODN, endotoxemia was induced by i.p. bolus of 25 mg/kg body weight LPS (=LD_{80}). Animals were monitored 7 days for mortality following LPS administration.

In a second series, 72 rats in 12 groups (Table 2) treated with 12 nmol i.p. CpG-ODN or control-ODN. 8 hours, respectively 7 days after administration of ODN, endotoxemia was induced by i.p. bolus of 10 mg/kg body weight LPS (=LD_0). Blood samples were drawn 3, 12 and 36 hours after LPS treatment. At each time point animals were sacrificed and organs were harvested, weighed and BAL fluid was immediately gained from the lungs. Blood samples for examination were gained from the heart of the animals.

Laboratory procedures

Blood samples

Blood cell counts (hemoglobin, hematocrit, erythrocytes, leukocytes, and platelets) were counted automatically (Blood Center, University Hospital of Bonn).

Measurement of BAL fluid IL-6 levels

Bronchoalveolar lavage was performed via tracheal cannulation using sterile normal saline. Five lavages of 8 ml each were used for a total recovered volume of approximately 30 ml. BAL fluid was centrifuged at 1000 rpm for 10 min. at 4°C and supernatants were stored at –30°C in aliquots. IL-6 was measured using a highly specific rat IL-6 ELISA (R&D Systems, Minneapolis, MN, USA).

Table 1. Experimental protocol 1

ODN type	time up to LPS treatment	12 nmol ODN	48 nmol ODN
CpG	8 hours	n=10	n=10
control		n=10	n=10
CpG	7 days	n=19	n=19
control		n=19	n=19

Table 2. Experimental protocol 2

ODN type	time up to LPS treatment	time points after LPS treatment (hours)		
		3	12	36
CpG	8 hours	n=6	n=6	n=6
control		n=6	n=6	n=6
CpG	7 days	n=6	n=6	n=6
control		n=6	n=6	n=6

Lung wet weight

Lung weights were measured on freshly dissected specimens free of other adherent tissues and cut from each main bronchus at the hilum. Wet weights were determined immediately. Wet weight to body weight ratio were calculated using initial body weight as the reference weight.

Histopathological examination

Harvested organs (lung, liver, spleen, kidney) were fixed in 4% buffered formaline. Total organs or parts were embedded in paraffine, sliced and stained with hematoxylin and eosin. An experienced pathologist then compared the slides histologically.

Statistical analyses

Statistical calculations were performed using SPSS 12.0 for Windows. All values are given as mean ± standard deviation. Differences in survival between control- and CpG-ODN treated groups were tested for significance using Chi square test. Wilcoxon U-test was used to compare the means of cytokine levels, blood counts, and organ weights between groups. ANOVA followed by *post hoc* LSD testing was performed to compare the means of cytokine levels and blood counts at different time points. P values of <0.05 were regarded as significant.

RESULTS

Mortality

Administration of different doses of CpG-ODN followed by an LD_{80} dose of LPS at different time points did not reveal a statistically relevant change of mortality. (CpG-ODN treated groups: 12/19, 13/19, 10/10, 9/10. Control-ODN treated groups: 13/19, 10/19, 8/10, 8/10.)

Blood cell counts

LPS caused a distinct increase of leukocytes post administration ($2.28*10^3$/µl to $9,45*10^3$/µl average of all groups, Figure 1a). In parallel with leukocytosis, platelets decreased rapidly in all experimental groups ($263.50*10^3$/µl to $53.55*10^3$/µl average of all groups, Figure 1b). These effects were unaffected by the pretreatment with CpG-ODN. Levels of hemoglobin, erythrocytes, and hematocrit remained unaltered for all experimental groups (data not shown).

IL-6 levels in BAL fluid

IL-6 was measured in all groups at time points 3, 12, and 36 hours after administration of LPS. IL-6 levels peaked at 3 hours (545.10 pg/ml average of all groups) and decreased at the 12 hour time point (173.78 pg/ml average of all groups). In all experimental groups, pretreatment with CpG-ODN did not affect cytokine production between the groups in a statistically relevant manner (Figure 2).

Lung wet weight

Lung wet weight could not detect differences between CpG-ODN animals and in controls (data not shown).

Histopathology

Besides the known histopathological changes in organs of LPS treated animals no differences compared to CpG-
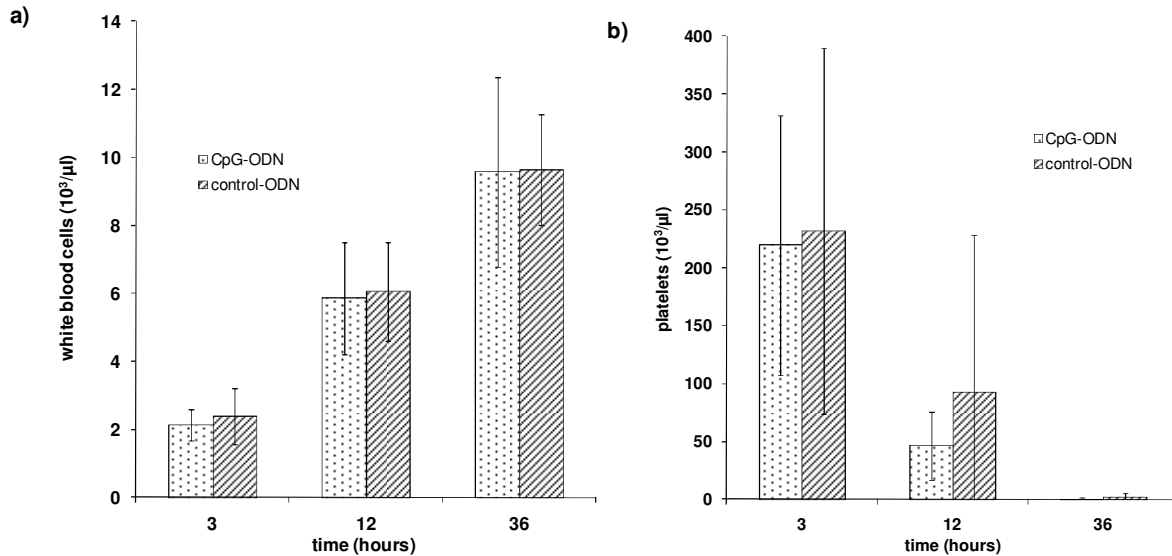
a)

b)

Figure 1. Leukocyte (a) and platelet (b) counts in CpG-ODN and control-ODN treated rats, followed by LPS treatment after 8 h. Data are means ± S.D.
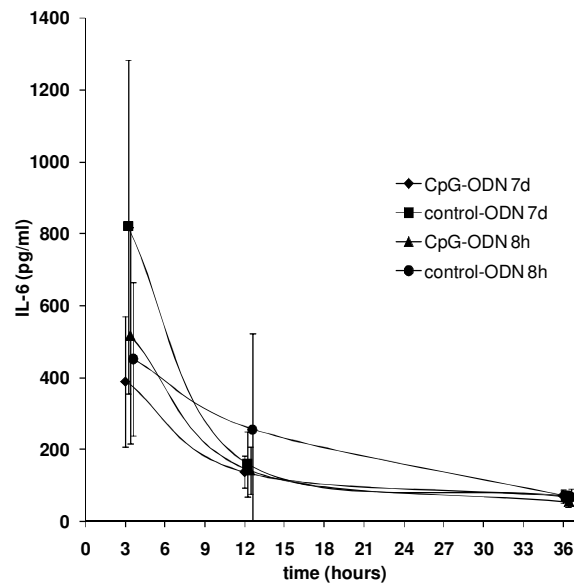
Figure 2. Effect of CpG-ODN on IL-6 levels in BAL fluid. Data are means ± S.D.

ODN pre-treated animals in lungs, liver, kidney and spleen could be detected.

DISCUSSION

The present study aimed to investigate whether administration of CpG-ODN could reduce endotoxin-induced mortality in rats and whether this effect might be attributable, at least in part, to a concomitant reduction in IL-6 levels in BAL fluid.

At all doses examined, CpG-ODN did not significantly affect mortality in an acute model of endotoxemia. Furthermore, application of CpG-ODN in the present model did not modify hematological parameters, histopathology, lung wet weight or stimulated IL-6 production in BAL fluid.

Within the last decade, much progress has been made in deciphering the proximal signaling pathways responsible for CpG-ODN mediated innate immune cell activation and the beneficial effects and therapeutic applications of CpG-ODN. Preliminary studies

demonstrated that CpG-ODN can be used for successful treatment of infections caused by bacteria, parasites or viruses (Dittmer and Olbrich, 2003). CpG-ODN has also been evaluated for the immunotherapy of cancer, including the treatment of kidney, skin, breast, uterine and immune malignancies (Jahrsdorfer and Weiner, 2003; Carpentier et al., 2003). Studies prove the beneficial effects in allergy by encouraging the development of T-helper 1 (T_h1) -type rather than T-helper 2 (T_h2) -type immune responses. Furthermore, CpG-ODN can act as vaccine adjuvant to improve antigen-presenting cell function and promote the induction of an antigen-specific adaptive immunity response (Klinmann, 2004). After successful phase I/II studies (Cooper et al., 2004), clinical phase III trials are pending to proof the therapeutic effect of CpG-ODN treatment on the bedside.

Beneficial effects of CpG-ODN may be explained by specific activation of innate immunity. The major difference of CpG-ODN compared to other PAMPs is the more specific pathway of inducing innate immune response via TLR-9. TLR-9 in humans is only expressed on B-cells and plasmacytoid dendritic cells (pDC) (Krieg, 2003). Following this activation the signaling pathway via the adaptor proteins MyD88, IL-1 receptor-activated kinase (IRAK) and tumor-necrosis factor receptor associated factor 6 (TRAF6), culminates in the activation of several transcription factors, including nuclear factor-κB (NFκB) and activating protein 1 (AP-1) (Haecker et al., 2002). These factors induce release of cytokines, which initiate a number of secondary effects, such as NK cell stimulation, maturation of pDCs and secretion of antibodies by B-cells. Together all these stimulated cells cause a T_h1-biased immune milieu. At this point the link between innate and adaptive immunity takes part. These effects might explain in part immunotherapeutic competence of CpG-ODN.

In contrast to immunomodulatory effects of CpG-ODN, interruption of the proximal signaling pathway might serve as an interesting approach to treat sepsis. The interaction between PAMPs, e.g. LPS, CpG-ODN and TLR alone demonstrate an induction of tolerance and cross-tolerance in many *in vitro* investigations. Sato and co-workers (Sato et al., 2000) reported synergy and cross-tolerance between TLR-2 and TLR-4 mediated signaling pathways. Dalpke and co-workers (Dalpke et al., 2005) examined tolerance induction in detail for Lipoteichoic acid (LTA), LPS and CpG-ODN. In RAW 264.7 macrophages, all three stimuli induced tolerance toward subsequent challenge with the same stimulus used for priming, as well as cross tolerance towards subsequent challenge with other stimuli via different TLR. However, pretreatment with CpG-ODN only protected against Galactosamine (GalN)/CpG-ODN challenge and failed to induce cross-tolerance for LPS and LTA *in vivo*.

TLRs differ in their requirements for signal transduction molecules. Due to this fact, activated signaling pathways have many similarities but also differences, e.g. MyD88-dependent and -independent signaling after TLR-3 and -4 activation (Dalpke et al., 2005). Sato and co-workers (Sato et al., 2002) states that macrophage-activating lipopeptide (MALP-2), while inducing cross-tolerance for MyD88-dependent LPS signaling, was not able to confer tolerance for the MyD88-independent LPS-specific signaling pathway. Although, TLR-7 only induces tolerance for MyD88-dependent LPS genes, while TLR-3-mediated cross-tolerance was restricted to MyD88-independent signaling. Other authors give credit that tolerance affects more genes than cross-tolerance (Dobrovolskaia et., 2003). This might be one reason for the lack of tolerance in the present study as well as in the other before named reports.

The transfer of *in vitro* as well as *in vivo* studies within different species is highly questionable due to varied expression of TLR-9 between species. In mice, immune cells of myeloid lineage express TLR-9, whereas in humans these cell types generally do not express TLR-9. Such differences are likely to result in marked differences in inflammatory mediator production. TLR-9 of different species diverged during the evolution. In consequence, the stimulatory potency of CpG motifs differs between species. Even the flanking regions of CpG motifs modify the effect (Klinmann, 2004).

In summary, we could not find any evidence for tolerances following endotoxemia induced by CpG-ODN pretreatment in the present *in vivo* study. These results demonstrate that in contrast to *in vitro* studies *in vivo* consequences greatly differ. Due to the complexity, single conclusion cannot be drawn and not be transferred on different protocols. Further studies are required to understand exactly the mechanisms of these contrasting data.

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