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Lipopolysaccharide contamination in intradermal DNA vaccination: Toxic impurity or adjuvant?

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ABSTRACT

Purpose: Lipopolysaccharides (LPS) are known both as potential adjuvants for vaccines and as toxic impurity in pharmaceutical preparations. The aim of this study was to assess the role of LPS in intradermal DNA vaccination administered by DNA tattooing.
Method: Mice were vaccinated with a model DNA vaccine (Luc-NP) with an increasing content of residual LPS. The effect of LPS on systemic toxicity, antigen expression and cellular immunity was studied.
Results: The presence of LPS in the DNA vaccine neither induced systemic toxicity (as reflected by IL-6 concentration in serum), nor influenced antigen expression (measured by intravital imaging). Higher LPS contents however, appeared to be associated with an elevated cytotoxic T-lymphocyte (CTL) response but without reaching statistical significance. Interestingly, the DNA tattoo procedure by itself was shown to induce a serum cytokine response that was at least as potent as that induced by parenteral LPS administration.
Conclusion: LPS does not show toxicity in mice vaccinated by DNA tattooing at dose levels well above those encountered in GMP-grade DNA preparations. Thus, residual LPS levels in the pharmaceutical range are not expected to adversely affect clinical outcome of vaccination trials and may in fact have some beneficial adjuvant effect. The observed pro-inflammatory effects of DNA tattoo may help explain the high immunogenicity of this procedure.

1. Introduction

Endotoxins, which are lipopolysaccharides (LPS) originating from gram-negative micro-organisms, are a common impurity in plasmid DNA (pDNA) preparations. This is inherently due to the fact that *E. coli*, a gram-negative bacterium, is used for amplification in the manufacturing process of the plasmid (Schleef and Schmidt, 2004; Przybylowski et al., 2007). In the down-stream processing of pDNA, *E. coli* cells are lysed, leading to high LPS levels originating from the outer cell membrane. LPS can be efficiently removed by different endotoxin removal steps and current protocols are capable of bringing LPS levels in pDNA preparations down to 0.01–100 International Units (IU)/mg, depending on the purification strategy applied (Horn et al., 1995; Montbriand and Malone, 1996; Marquet et al., 1997; Ferreira et al., 2000; Schalk et al., 2001; Quaak et al., 2008).

Residual LPS in pDNA drug products can be potentially toxic, as parenteral injection of LPS can result in a septic shock like syndrome (Bentala et al., 2002; Copeland et al., 2005). Therefore, a limit on maximal endotoxin dose for injectable dosage forms has been established (<5 IU/kg of body mass per hour (European Pharmacopoeia; US Pharmacopeia).

Because of its immunostimulatory properties, LPS is also seen as a potential adjuvant for vaccines (Hawkins et al., 2003). LPS can activate and mature dendritic cells (DCs) *in vitro* (Granucci et al., 1999) and *in vivo* (Michelsen et al., 2001) by triggering Toll-like receptor (TLR) 4 (Qureshi et al., 1999; Backhed et al., 2003). DCs play an important role in vaccination, as they serve as professional antigen presenting cells (APCs), necessary for antigen presentation and the initiation of adaptive immunity (Thurnher, 2007). To benefit from the adjuvant properties of LPS without encountering systemic toxicity, a detoxified form of Lipid A derived from LPS (MonoPhosphoryl Lipid A; MPLA) has been developed, and is already successfully used

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as an adjuvant in vaccine formulations (Sasaki et al., 1997; Freytag and Clements, 2005).

Our institute recently described a novel method for intradermal DNA vaccination, called DNA tattooing. This vaccination strategy is able to induce fast cellular immune responses upon immunization with pDNA in mice and outperforms classical intradermal DNA vaccination by 10–100-fold in non-human primates (Bins et al., 2005; Verstrepen et al., 2008). Since DNA tattoo vaccination relies on the use of a needle array for administration, we consider this technique as a parenteral administration route LPS levels in vaccine batches produced for clinical administration should be limited (Quaak et al., 2008). In order to unravel the role of LPS in DNA vaccines applied by tattooing, the first aim of this study was to assess the toxicity of LPS when using this delivery technique. The second aim was to study the influence of LPS on the magnitude of the antigen-specific T-cell responses induced by DNA tattoo vaccination.

Together, these results should provide information on the role of LPS during pre-clinical and clinical intradermal DNA tattoo vaccination, both from a pharmaceutical as well as from an adjuvant perspective.

2. Materials and methods

2.1. Mice

C57BL/6J mice (2–4 months) were obtained from the experimental animal department of The Netherlands Cancer Institute. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Institute's Animal Research Committee.

2.2. DNA vaccines

To measure *in vivo* antigen expression and cellular immune responses, a model plasmid DNA, pVAX:Luc-NP (Bins et al., 2005), was used which encodes the influenza A $NP_{366–374}$ epitope fused to the carboxy terminus of firefly luciferase inserted into the EcoRI/NotI sites of the pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). Plasmid DNA was expressed and amplified in *E. coli* DH5. DNA was purified using either the QIAGEN Mega-kit or the Endofree™ QIAGEN Mega-kit (QIAGEN, Hilden, Germany) which resulted in plasmid preparations containing 5000 and 2 IU/mg pDNA respectively as determined in duplicate by a Pyrochrome® limulus amoebocyte lysate assay (Cape Cod Associates, Cape Cod, MA, USA). Both preparations were mixed in different ratios to obtain the required range of LPS concentrations.

2.3. DNA immunization and control groups

For intradermal DNA vaccination, 10 μl of a 2 mg/ml DNA solution in Water for Injection (Wfl) was applied to the shaved left hind leg of the mice and administered using a disposable 9-needle cartridge (MT Derm, Berlin, Germany) mounted on an Aella® tattoo machine for medical use (MT Derm). DNA vaccines were tattooed during 30 s at a needle depth of 1.0 mm, with an oscillation frequency of 100 Hz. Mice were vaccinated using a standard schedule on day 0, 3 and 6 (Bins et al., 2005) with DNA solutions containing increasing amounts of LPS (2, 100, 500 and 5000 IU/mg DNA), corresponding to an absolute amount of 0.04, 2, 10 and 100 IU LPS/dose of 20 μg plasmid.

As a positive control for IL-6 release, mice were injected intraperitoneally with 10 ng (100 IU) LPS from *E. coli* 0113:H10 (Cape Cod Associates, Cape Cod, MA, USA) diluted in 200 μl of Phosphate Buffered Saline (PBS).

As negative controls, mice were tattooed with Wfl or were sham-treated. Sham-treated mice were shaved and anesthetized, but the mice did not receive any tattoo or injection. Table 1 shows the different groups used in this study. All groups were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA) during treatment. At the indicated time points post-immunization, approximately 50 μl of peripheral blood was collected by tail bleeding for the measurement of serum IL-6 levels, and antigen-specific T-cell responses.

2.4. IL-6 ELISA

The concentration of the pro-inflammatory cytokine IL-6 in serum was measured using an IL-6 ELISA kit (R&D systems, Minneapolis, MN USA). Serum samples were diluted 1:10 with PBS in order to keep the results within the linear range of the assay, as determined by the calibration curve. The sensitivity of the assay was 1.6 pg/ml according to the calibration curve.

2.5. Antigen expression using intravital imaging

To monitor antigen expression upon DNA vaccination, a light-sensitive camera was used for longitudinal measurement of *in vivo* firefly luciferase activity. Mice were anesthetized with isoflurane. A solution of the firefly luciferase substrate luciferin in PBS (150 mg/kg, Xenogen, Hopkinton, USA) was injected intraperitoneally and 18 min later the luminescence produced by active luciferase was acquired during 30 s in an IVIS® system 100 CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

2.6. Cytotoxic T-cell assay

To measure Cytotoxic T-Lymphocyte (CTL) responses, peripheral blood lymphocytes were stained with PE-conjugated H-$2D^b$/$NP_{366–374}$-tetramers and APC-conjugated CD8α antibody (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1× PBS, 0.5% BSA and 0.02% sodium azide) as described previously (Toebes et al., 2006). Subsequently, cells were washed three times in FACS buffer and analyzed by flow cytometry. Living cells were selected based on propidium iodide exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton

Table 1
Group categorization. Abbreviations: LPS, lipopolysaccharide; IU, International Units; Wfl, Water For Injections; i.p., intraperitoneal.

Group	Compound	Administration route	Amount LPS	Number of animals
1	Luc-NP DNA vaccine 20 μg	Tattoo	2 IU/mg DNA (0.04 IU/dose)	6
2	Luc-NP DNA vaccine 20 μg	Tattoo	100 IU/mg DNA (2 IU/dose)	6
3	Luc-NP DNA vaccine 20 μg	Tattoo	500 IU/mg DNA (10 IU/dose)	6
4	Luc-NP DNA vaccine 20 μg	Tattoo	5000 IU/mg DNA (IU/dose)	6
Wfl	Wfl 10 μl	Tattoo	<0.01 IU/ml[a] (<2×10^{-4} IU/dose)	4
i.p. (positive control)	Purified LPS	i.p. injection	100 IU/dose	4
Sham (negative control)	–	–	–	4

[a] According certificate of analysis.

Dickinson, Franklin Lakes, USA) using Summit analysis software (Dako, Glostrup, Denmark).

2.7. Statistical analysis

Data were analyzed using a ANOVA and Tukey's test and P values <0.05 (tested two-sided) were considered statistically significant.

3. Results and discussion

The aim of the present study was to investigate the role of LPS in genetic vaccination by DNA tattoo administration. Since LPS is a potent immunostimulator (Bentala et al., 2002; Copeland et al., 2005) the effect of LPS on toxicity, antigen expression and cellular immunity was investigated. For this purpose, mice were immunized with a construct encoding the fusion protein of luciferase and a MHC class I restricted influenza A NP epitope (Luc-NP), to allow the simultaneous measurement of antigen expression and vaccination-induced T-cell responses. DNA vaccines were prepared with increasing amounts of residual LPS, ranging between 2 IU/mg (highly purified) and 5000 IU/mg (unpurified product). 100 IU/mg was chosen as the pharmaceutical upper level and 500 IU/mg as intermediate LPS content. The pharmaceutical upper level was set based on an average patient weight of 70 kg. A patient of that weight is allowed to receive a maximum of 70 kg × 5 IU/kg/h = 350 IU LPS per hour. As all DNA vaccine doses are normally administered within an hour, an LPS content of 100 IU/mg pDNA allows a maximum of 3.5 mg pDNA/dose. This maximum injectable dose of 3.5 mg pDNA is sufficient to cover current intradermal clinical DNA vaccination protocols (Mincheff et al., 2000; Timmerman et al., 2002; Rosenberg et al., 2003; Pavlenko et al., 2004; Miller et al., 2005).

3.1. Effect of LPS on serum IL-6

To determine the systemic toxicity of LPS, serum IL-6 levels of the treated animals were measured using an IL-6 ELISA, the first 24 h after administration. It is known that systemic administration of LPS results in the production of pro-inflammatory cytokines, such as IL-6, by activated APCs. Furthermore, these cytokines are important mediators in the fever-like response upon LPS administration (Copeland et al., 2005; Harden et al., 2006) and therefore a good predictor for toxicity.

Mice receiving an intraperitoneal injection of 100 IU LPS (positive control) showed a peak in IL-6 concentration 2 h after injection, similar in kinetics and magnitude as reported previously (Fig. 1) (Copeland et al., 2005; Zhang et al., 2007). The sham-treated group (negative control) showed a small rise in serum IL-6 levels on the day of treatment, reflecting the effect of handling stress, including anaesthesia, shaving and tail bleedings (Nukina et al., 2001).

The control group which received a tattoo with Wfl showed a strong increase in IL-6 serum levels, with a peak 7 h after administration, presumably caused by damage of the skin by tattooing. It is possible that the measured IL-6 is released by damaged keratinocytes, since it is known that these cells can release IL-6 upon stress exposure (Yoshizumi et al., 2008). All mice receiving DNA tattoo vaccines containing increasing amounts of LPS showed the same, tattoo-associated, serum IL-6 peak, 7 h after administration. Importantly, tattoo administration showed no differences in both kinetics and magnitude of IL-6 serum level between the different amounts of LPS. Moreover, none of the mice showed any physical signs of illness. This suggests that even the highest concentration of LPS is not associated with an increase in systemic toxicity for this intradermal route of genetic vaccination.

These observations showed that high levels of LPS in dermal vaccines applied by DNA tattooing does not result in additional release of IL-6 above the release already provoked by the tattooing technique itself. It is important to emphasize that humans are 250 times more sensitive to a systemic LPS challenge than mice, based on acute inflammatory response (Copeland et al., 2005). However, since patients will be vaccinated with purified pDNA with LPS levels ≤2 IU/mg, inflammatory reactions provoked by this concentration of residual LPS are unexpected, since mice vaccinated with 250–2500-fold higher levels (500 and 5000 IU/mg) did not show any additional IL-6 release in this experiment either.

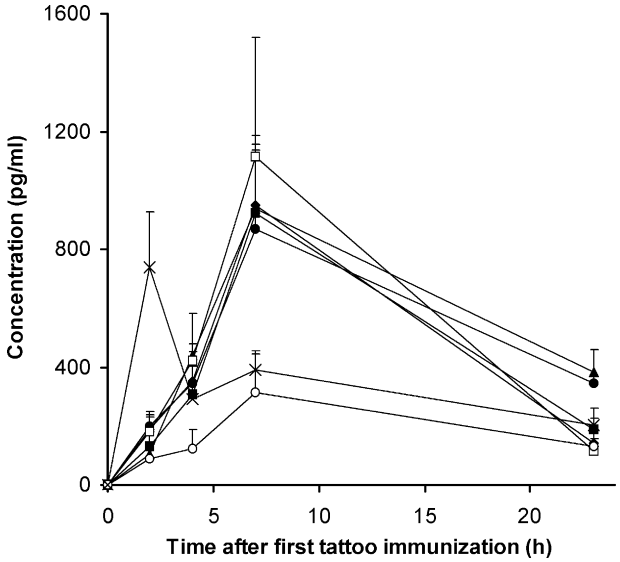

Fig. 1. IL-6 response in serum after DNA tattoo immunization. Cohorts of mice ($n = 6$) were immunized by DNA tattooing with the Luc-NP vaccine batches containing an increasing amounts of LPS (2 IU (▲), 100 IU (■), 500 IU (●) or 5000 IU(♦)/mg pDNA). As controls, cohorts of mice ($n = 4$) received a tattoo with Wfl (□), 100 IU purified LPS intraperitoneally (x) or were sham-treated (○). Values represent the mean + standard error of the mean (error bar) for each treatment group.

3.2. Effect of LPS on antigen expression

To study the effect of LPS on *in vivo* antigen expression, luciferase expression was measured with a light sensitive CCD camera on day 1 and 3 after DNA tattooing with the Luc-NP construct. All groups showed a similar luciferase expression profile upon vaccination (Fig. 2) with no significant effect of administered LPS concentration on luciferase expression.

In literature, the presence of LPS is associated with a decrease in transfection efficiency of pDNA in eukaryotic cells *in vitro* (Weber et al., 1995; Poxon and Hughes, 1999) and *in vivo* (Budryk et al., 2001). In addition, it is known that very high LPS concentrations (26,000 IU/mg pDNA) can inhibit DNA vaccination-induced gene expression after intratumoral injection in mice (Budryk et al., 2001). However, at the LPS levels used in this study, no substantial effect of LPS on intradermal antigen expression was observed.

3.3. Effect of LPS on CTL response

The effect of LPS on cellular immunity was assessed by determining the percentage of $NP_{366-374}$ specific $CD8^+$ T-cells in peripheral blood at the peak of the DNA vaccine-induced T-cell responses, 13 days after start of vaccination (Bins et al., 2005). All mice showed a strong epitope specific T-cell response upon DNA tattooing (Fig. 3). Mice receiving vaccinations with a high content of LPS (500 or 5000 IU/mg pDNA) showed a higher, but also a more variable CTL response. ANOVA analysis showed only a significant relationship ($p < 0.05$) between de groups receiving 2 and 500 IU/mg.

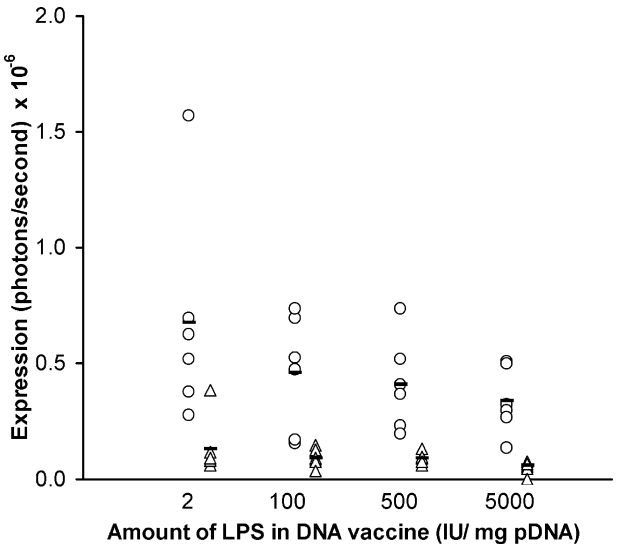

Fig. 2. Effect of LPS content on *in vivo* antigen expression level after DNA tattoo immunization, as determined by a light-sensitive camera on days 1 (○) and 3 (Δ). Antigen expression levels of individual mice are plotted together with group averages (—).

LPS is widely studied as an adjuvant for DNA vaccination. It has previously been shown that LPS is able to increase IgG responses upon intradermal injection with a DNA vaccine, without affecting CTL responses (Boyle et al., 1998). Previous experiments with intradermal gene-gun DNA vaccination did not show a relationship between CTL responses and LPS level either (Hawkins et al., 2003). In that study, LPS levels up to 56,000 IU/mg pDNA were tested. Our results show that high concentrations of LPS in pDNA vaccines for tattoo-administration might act as an adjuvant, associated with a higher CTL response, but lacks statistical power to validate this finding. Importantly, LPS variations within the pharmaceutical spectrum (between 2 and 100 IU/mg pDNA) do not have any beneficial or toxic effects when using dermal tattoo administration. This means that batch-to-batch variations in LPS level within pharmaceutical accepted ranges in pDNA products will probably not influence the outcome of clinical trials.

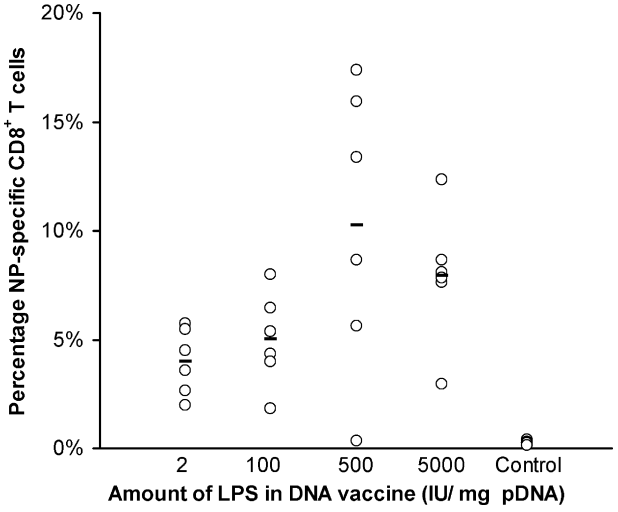

Fig. 3. NP_{366}-specific T-cell responses in cohorts of mice ($n = 6$) upon tattoo vaccination on day 0, 3 and 6 with Luc-NP vaccine batches containing an increasing amount of LPS per dose of pDNA. Control mice were tattooed with Wfl ($n = 4$). NP_{366}-specific T-cell responses were determined on day 13 by MHC tetramer staining of peripheral blood lymphocytes. The percentage of NP_{366}-reactive $CD8^+$ T-cells of total $CD8^+$ T-cells for individual mice is plotted (○) together with the group averages (—).

We have previously established procedures for the large-scale production of clinical grade pDNA under GMP conditions (Quaak et al., 2008). By means of an endotoxin removal step, the endotoxin level of the pDNA produced is reduced to ≤2 IU/mg. In a recently started clinical trial pDNA dose will be escalated from 0.5 to 8 mg, corresponding to administration of a maximum of 1–16 IU LPS/dose. Based on the data presented here, this amount of LPS will, most probably, not affect toxicity, expression or immunogenicity of the administered pDNA.

A remarkable observation in this study was that the tattooing procedure itself resulted in a systemic IL-6 release that reached higher concentrations than an intraperitoneal injection with LPS. Possibly, this IL-6 release is an explanation for the high immunogenicity of DNA vaccines administered by tattooing.

In conclusion, in this specific route of pDNA vaccine administration, LPS serves as a non-toxic contaminant. However, LPS concentrations above pharmaceutical limits have the potential to act as adjuvant. In addition, the results of the present study might also be extrapolated to other dermal DNA vaccination techniques, such as jet injector, gene gun, microneedles or electroporation.

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