Chapter 5

Consequences of the expression of lipopolysaccharide-modifying enzymes for the efficacy and reactogenicity of whole-cell pertussis vaccines

Jeroen Geurtsen, Rob J. Vandebriel, Eric R. Gremmer, Betsy Kuipers, Jan Tommassen, and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial outer membrane and is, due to its endotoxic activity, responsible for the relatively high reactogenicity of whole-cell vaccines. In addition, LPS has strong immune stimulating properties, which makes it, potentially, an interesting vaccine component. In a previous study, we have shown that expression of two LPS-modifying enzymes, i.e., PagP and PagL, modulates the endotoxic activity of the Gram-negative bacterium *Bordetella pertussis*, the causative agent of whooping cough. To assess the consequences of PagP and PagL expression on the efficacy and reactogenicity of whole-cell pertussis (wP) vaccines, we have immunised mice and challenged them intranasally with wild-type *B. pertussis*. Vaccine efficacy, *B. pertussis*-specific antibody responses, and cytokine profiles were evaluated. The results show that expression of PagL, but not of PagP, significantly increases vaccine efficacy without altering vaccine reactogenicity. Therefore, PagL-expressing *B. pertussis* strains may form a basis for the development of a new and safer wP vaccine, as higher vaccine efficacies may allow a reduced vaccine dosage. These data show, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

Introduction

Pertussis or whooping cough is a severe acute respiratory illness that is characterised by paroxysmal coughing and a distinctive “whooping” sound when air is subsequently inhaled. The disease is highly contagious and most severe in neonates and children younger than one year. Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*, which was first isolated in 1906 by Bordet and Gengou (Bordet and Gengou, 1906).

Introduction of whole-cell pertussis (wP) vaccines in the 1940s and 1950s led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after their introduction, it became clear that wP vaccines exhibited a considerable reactogenicity, which was caused by the presence of strong immunogens, such as lipopolysaccharide (LPS) and pertussis toxin. Therefore, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the 1980s and 1990s. Today, aP vaccines are broadly used in industrialised countries and have been shown to be highly effective. Nevertheless, aP vaccines have, as compared to wP vaccines, some important disadvantages. These include an increased cost/benefit ratio, which prohibits their use in the developing world, the loss of the adjuvant effect of the wP vaccines, and the fact that aP vaccination skews towards a Th2 immune response, whereas wP vaccination and natural infection have been shown to evoke more of a Th1 immune response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998; van den Berg *et al.*, 2001). For these reasons, the development of new and safer wP vaccines remains an important issue.

An important factor that contributes to the relatively high reactogenicity of wP vaccines is the presence of LPS. LPS is one of the major constituents of the Gram-negative bacterial outer membrane, where it is important for maintaining the membrane barrier function. LPS is an amphiphatic molecule that consists of three domains, i.e., the O-antigen, the core, and lipid A (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also possesses a powerful adjuvant activity. Both these properties are based upon the recognition of the LPS by the host TLR4/MD-2 receptor complex and the subsequent activation of NF-κB (reviewed in O’Neill, 2006; Pålsson-McDermott and O’Neill, 2004).

The endotoxic activity of LPS is largely determined by the composition of its lipid A moiety (Homma *et al.*, 1985). In general, lipid A consists of a β-1,6-linked D-glucosamine disaccharide carrying phosphate groups at positions C-1 and C-4’ and ester- and amide-linked 3-hydroxy fatty-acids (Raetz and Whitfield, 2002). Previous

studies have shown that the phosphate groups, as well as the number and the length of the acyl chains are critical determinants of the endotoxic activity of lipid A and that changes in lipid A composition often modulate its biological activity (Geurtsen *et al.*, 2006; Loppnow *et al.*, 1989; Steeghs *et al.*, 2002; van der Ley *et al.*, 2001).

After injection into mice, LPS rapidly induces increased serum pro-inflammatory cytokine levels (Durand *et al.*, 2004). Pro-inflammatory cytokines have been shown to play a central role in the systemic inflammatory effects elicited by a variety of Gram-negative bacteria, including *B. pertussis* (Kutukculer *et al.*, 1997; Loscher *et al.*, 2000; Sawa *et al.*, 1997). Furthermore, pro-inflammatory cytokines have been associated with the relatively high reactogenicity of wP vaccines (Armstrong *et al.*, 2003; Loscher *et al.*, 1998). Hence, a straightforward approach to reduce wP reactogenicity would be the generation of *B. pertussis* strains that contain LPS with reduced endotoxic activity. Such strains could then function as a basis for safer wP vaccines. Of course this approach is only feasible when the second important property of the LPS, i.e., its adjuvant activity, is retained.

In a previous study, we have shown that expression of two lipid A-modifying enzymes, i.e., PagP and PagL, modulates the endotoxic activity of *B. pertussis* (Geurtsen *et al.*, 2006). Expression of PagP, a lipid A acylase, increased the endotoxic activity of both LPS and whole bacterial cells, whereas expression of PagL, a lipid A 3-*O*-deacylase, decreased the endotoxic activity of the purified LPS, but increased the toxicity of the whole bacterial cells. This latter observation was rather unexpected and might be related to an increased release of the deacylated LPS from the bacterial membranes (Geurtsen *et al.*, 2006). In the study presented here, we evaluated the consequences of LPS acylation or deacylation on the vaccine potential of wP vaccines. We immunised mice with wP vaccines based upon wild-type *B. pertussis* or *B. pertussis* expressing either PagP or PagL and subsequently analysed vaccine efficacy, *B. pertussis*-specific antibody responses, and cytokine profiles.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise notified, *B. pertussis* strain B213, a streptomycin resistant derivative of *B. pertussis* strain Tohama (Kasuga *et al.*, 1953), and its plasmid-containing derivatives were grown at 35°C on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood (Tritium, Veldhoven, the Netherlands). When appropriate, 100 μg/ml ampicillin was added to the medium for plasmid maintenance.

Vaccine preparation

Vaccines were prepared from wild-type *B. pertussis* strain B213 harbouring the empty pMMB67EH vector, plasmid pMMB67EH-PagP$_{(Ec)}$, containing the *Escherichia coli pagP* gene (Tefsen *et al.*, 2005), or pMMB67EH-PagL$_{(Bb)}$, containing the *Bordetella bronchiseptica pagL* gene (Geurtsen *et al.*, 2006). The bacteria were grown in synthetic THIJS medium (Thalen *et al.*, 1999) supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 68 h at 35°C while shaking (175 rpm). The bacterial cell suspensions were heat inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100 x *g* and resuspended in phosphate-buffered saline (PBS) to an A_{590} of 2.5, i.e., 50 international opacity units (IOU). The suspensions were stored at 4°C. Prior to immunisation, the suspensions were further diluted in PBS to final concentrations of 3.2 IOU/ml or 16 IOU/ml (depending on the experiment), after which 0.3 mg/ml or 1.5 mg/ml aluminum phosphate (final concentration), respectively, was added as an adjuvant. The resulting vaccine concentrations corresponded to 1/10 and 1/2 human dose per 0.5 ml, respectively. Furthermore, Western blot analysis showed that the vaccines contained similar amounts of the virulence factors pertactin, fimbriae, pertussis toxin, and filamentous haemagglutinin (data not shown).

Immunisation and intranasal challenge

All animal experiments were performed at the Netherlands Vaccine Institute in accordance with the Dutch national guidelines for animal experimentation. Groups of nine 4- to 5-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml vaccine (prepared as described above), or with 0.5 ml PBS/$AlPO_4$ as a control, at days 0 and 14. At day 28, the mice were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *B. pertussis* strain B213 in 40 μl medium.

Autopsy

The left lung lobes were collected in 1 ml Verwey medium and homogenised using a tissue homogeniser (Pro-200, ProScientific, Monroe, CT, USA) at maximum speed for 10 s. The homogenates were diluted 10- and 100-fold for the immunised mice and 1000-fold for the control mice, and 100-μl aliquots of the dilutions were plated on BG plates supplemented with 30 μg/ml streptomycin and incubated at 35°C for 5 days. Results are reported as log protection (LOG_{Prot}) values, which allows for comparison of vaccine efficacies. The LOG_{Prot} was calculated using the following equation: LOG_{Prot} =

^{10}log (mean CFU of PBS/AlPO$_4$-treated mice) - ^{10}log (CFU of each individual immunised mouse). Blood was collected 4 h post immunisation at days 0 and 14 for cytokine analysis and at day 28 for the assessment of antibody titers.

Combined mouse toxicity test

The combined mouse toxicity test was performed as described (van Straaten *et al.*, 2002). In short, groups of 10 male N:NIH/RIVM outbred mice (14-17 g) were immunised intraperitoneally with 0.5 ml wP vaccine (1/2 human dose), or with 0.5 ml PBS/AlPO$_4$ as a control. Before immunisation, the mice were distributed randomly in groups of five per cage and weighed individually. Sixteen h after immunisation, the mice were weighed again. On day 7 after immunisation, a 10 μl blood sample was taken from the tail vein, using an EDTA-impregnated capillary, diluted in 1.6 ml Isoton (Coulter), after which the number of leukocytes was determined using a Coulter Counter.

Antibody titer determination

Total IgG, IgG1, and IgG2a antibody titers against whole cells of *B. pertussis* strain B213 were determined in an enzyme-linked immunosorbent assay (ELISA) as described (Abdillahi and Poolman, 1987). In short, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100-μl samples of a whole-cell suspension (A_{590} = 0.05 in PBS). Antibody titers were measured for individual sera using HRP-labelled goat anti-mouse secondary antibodies (SouthernBiotech). A four-parameter curve fit was made for optical density values of 3-fold serial dilutions, and the antibody titers were calculated in reciprocal dilutions that gave 50% of the maximum absorbance.

Cytokine determination

Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) concentrations in the sera were quantified with an ELISA against mouse IL-6 or TNFα according to the manufacturers' instructions (eBioscience). For determination of cytokine concentrations in the supernatant of lung homogenates, an 11-plex panel containing beads for mouse IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-α, TNFα, and GM-CSF (Bio-Rad, Hercules, CA) was used. 96-wells filter bottom plates (Bio-Rad) were pre-wet with 100 μl of Bio-Plex assay buffer (Bio-Rad). After each step, buffer was removed by vacuum. Beads were diluted in assay buffer, and 50 μl of the solution was added per well. The plates were washed twice with 100 μl of Bio-Plex wash buffer (Bio-Rad). The cytokine standards were diluted in Verwey medium to a range of 32,000 to 0.18 pg/ml. Of

standards and samples, 50 μl/well were added. The plates were incubated for 30 min; each incubation step consisted of vortexing the plates at 1100 rpm for 30 s, followed by incubation with shaking at 300 rpm. The plates were washed 3 times with 100 μl of wash buffer. Detection antibody was diluted in detection antibody diluent (Bio-Rad), and 25 μl/well was added. The plates were incubated for 30 min, and washed 3 times with 100 μl of assay buffer. Streptavidin-phycoerythrin was diluted in assay buffer, and 50 μl/well was added. The plates were incubated for 10 min, and washed 3 times with 100 μl of wash buffer. The beads were resuspended in 125 μl of assay buffer and analysed on a Bio-Plex (Bio-Rad). The results shown were obtained at low photo multiplier tube settings. All steps were performed at room temperature.

Statistical analysis

Data were statistically analysed using one-way analysis of variation (ANOVA) followed by Bonferroni's multiple comparison test. Differences were considered to be significant when $p < 0.05$.

Results

Protection against *B. pertussis* challenge after subcutaneous immunisation with whole-cell pertussis vaccines

To evaluate the protection offered by wP vaccines based upon wild-type *B. pertussis* strain B213 or its derivatives expressing *E. coli* PagP or *B. bronchiseptica* PagL, 4/5-wk-old BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a human dose of the vaccines and subsequently challenged with wild-type *B. pertussis* strain B213. All three vaccines conferred significant protection against colonisation by the wild-type strain. When mutually compared, immunisation with the PagL-expressing strain (LOG_{Prot}~2.8) provided significantly better protection against a challenge than did immunisation with the wild-type strain (LOG_{Prot}~1.5) (Fig. 1). Also immunisation with the PagP-expressing strain provided a better protection (LOG_{Prot}~2.2), but in this case the difference was not significant (Fig. 1). Thus, the results indicate that the vaccine consisting of the PagL-expressing strain had a significantly higher efficacy. Of note, the decision to use *E. coli* PagP in stead of *Bordetella* PagP for our study was based upon our previous finding that a *B. pertussis* strain expressing the latter enzyme showed only poor modification efficiency (Geurtsen *et al.*, 2006).

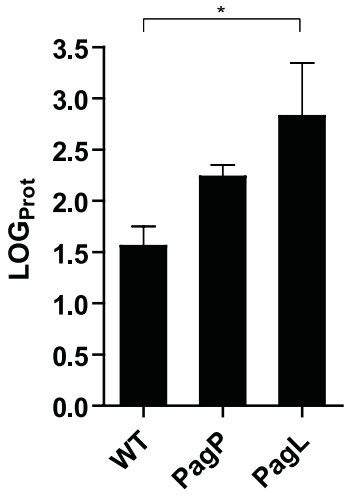

Fig. 1. Efficacy of whole cell pertussis vaccines in a mouse model. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT) or *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL) and challenged with wild-type *B. pertussis* at day 28. At day 5 after challenge, lung CFU numbers were determined. Log protection values were calculated by subtracting the Log10 CFU of individual mice from the mean Log10 CFU of the PBS/$AlPO_4$-treated mice. Results are expressed as mean log protection values (± SEM) from nine mice per group and are representative of three separate experiments. A single asterisk marks significant ($p<0.05$) differences.

B. pertussis specific antibody responses

The *B. pertussis*-specific total IgG, IgG1, and IgG2a serum antibody titers at day 28 after primary immunisation were determined in whole-cell ELISAs. High *B. pertussis*-specific serum IgG titers were elicited with all three vaccines (Fig. 2A). As compared to the mice immunised with the wild-type strain, the mice immunised with the PagP- and PagL-expressing strains showed similar IgG titers (Fig. 2A). Subsequent analysis of the IgG1/IgG2a subclass distribution showed similar IgG1 titers in all groups, although the titers in the group immunised with the PagL-expressing strain were slightly higher. The PagP- and PagL-expressing strains both elicited a higher IgG2a response than did the wild-type strain (Fig. 2B). A decreased IgG1/IgG2a ratio in the groups immunised with the PagP- and PagL-expressing strains suggests that these animals generally exhibited stronger Th1-type responses than the mice immunised with the wild-type strain.

Cytokine profiles in the lung

The supernatants of lung homogenates prepared at day 5 after challenge were assessed for their cytokine concentrations (Fig. 3). As compared to the PBS/$AlPO_4$ control group, the IL-1α level was significantly higher in the lungs of the mice immunised with the PagL-expressing strain, but not in those of the mice immunised with the wild-type or the PagP-expressing strain (Fig. 3A). In addition, the lungs of the mice immunised with the PagL-expressing strain showed a significantly higher IFN-γ level, as compared to all other groups (Fig. 3B). Analysis of the other cytokines, i.e., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNFα, and GM-CSF, did not reveal significant differences compared to the control group.

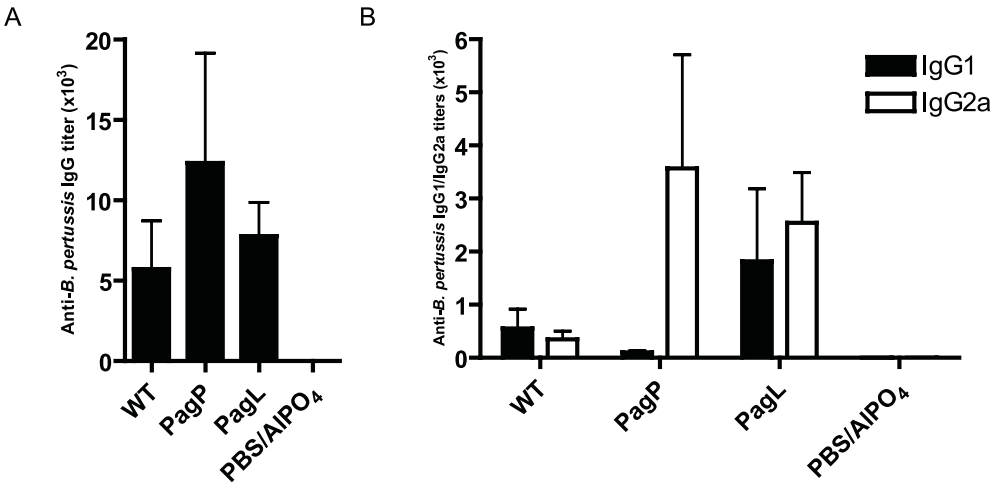

Fig. 2. *B. pertussis*-specific antibody responses. Anti-*B. pertussis* IgG (A) and IgG1/IgG2a (B) titers at day 28 post primary immunisation were measured in mice immunised with wP vaccines based upon *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control using a whole-cell ELISA. Results are expressed as mean antibody titers (± SEM) from nine mice per group and are representative of three separate experiments.

Pro-inflammatory cytokines in serum

To address the *in vivo* reactogenicity of the vaccines, we analysed the concentration of two pro-inflammatory cytokines, i.e., TNFα and IL-6, in serum samples taken 4 h after the primary or booster immunisation (Fig. 4). As shown in Fig. 4A, serum TNFα levels were similar in all groups and no significant differences were found. On the other hand, all three wP immunisations elicited significantly higher serum IL-6 levels than did the PBS/$AlPO_4$ control (Fig. 4B). Comparison of the IL-6 levels between the wP vaccinated groups showed no significant differences, indicating that the reactogenicity of the three wP vaccines, as assessed by this assay, was comparable.

Vaccine toxicity test

To obtain more insight into the overall toxicity displayed by the vaccines, a mouse weight gain test in combination with a leukocytosis promotion test was performed. The first test assesses the weight gain of individual mice at 16 h post immunisation and provides a general measure for vaccine toxicity, whereas the leukocytosis promotion test, in which

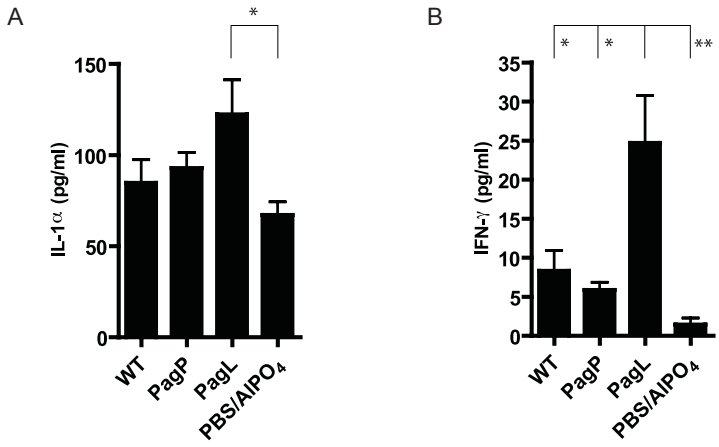

Fig. 3. Lung cytokine concentrations at day 5 post challenge. Mice were immunised with wP vaccines based upon *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control and challenged with wild-type *B. pertussis*. At day 5 after challenge, IL-1α (A) and IFN-γ (B) concentrations in the lung-homogenate supernatant were measured. Results are expressed as mean cytokine concentrations (± SEM) from six mice per group and are representative of two separate experiments. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

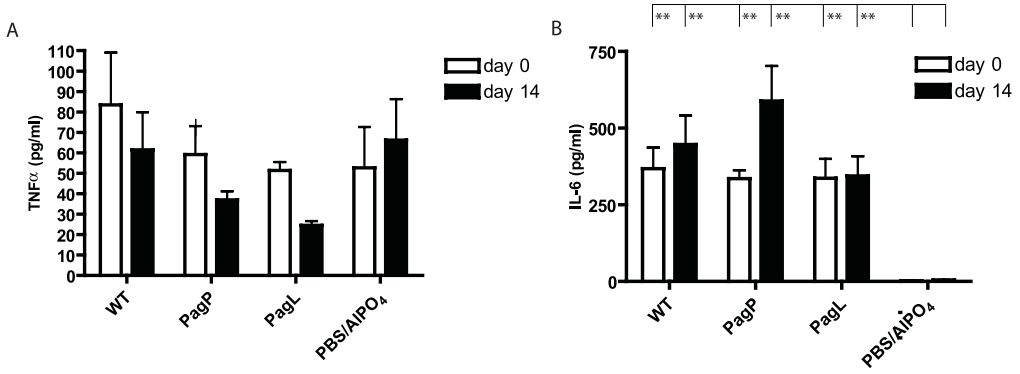

Fig. 4. Serum pro-inflammatory cytokine concentrations 4 h post immunisation. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control. Serum TNFα (A) and IL-6 (B) concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from nine mice per group and are representative of three separate experiments. Double asterisks mark highly significant ($p<0.001$) differences.

the numbers of leukocytes in the blood are determined at day 7 after immunisation, specifically assesses pertussis toxin-mediated leukocytosis (van Straaten *et al.*, 2002). As shown in Fig. 5A, immunisation with the wP vaccines resulted in a substantial loss of weight 16 h post immunisation, which is consistent with the relatively high toxicity of wP vaccines. When the wP vaccines were mutually compared, no significant differences in vaccine toxicity were found. However, as compared to the other vaccines, the mice immunised with the PagP-expressing strain exhibited significantly increased leukocyte counts in their blood (Fig. 5B), which indicates that in this particular vaccine, the activity of pertussis toxin may be enhanced.

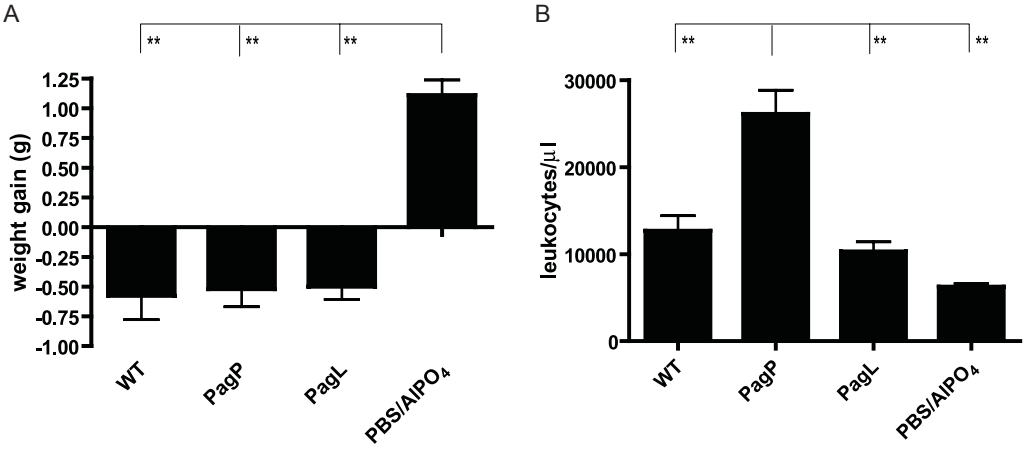

Fig. 5. Assessment of vaccine reactogenicity in a vaccine toxicity test. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control and the weight gain 16 h post immunisation (A) or the number of leukocytes in the blood at day 7 (B) were determined. Results are expressed as mean weight gain or mean leukocyte number per µl blood (± SEM) from nine mice per group. Double asterisks mark highly significant ($p<0.001$) differences.

Discussion

The goal of this study was to evaluate the effects of the expression of two LPS-modifying enzymes, i.e., *E. coli* PagP and *B. bronchiseptica* PagL, on the efficacy and reactogenicity of wP vaccines in a mouse model. In a previous study, we have shown that expression of these enzymes in *B. pertussis* modulates the toxicity of both LPS and whole bacterial cells (Geurtsen *et al.*, 2006). Here, we showed that immunisation with either wild-type *B. pertussis* or *B. pertussis* expressing PagP or PagL conferred significant protection against a subsequent challenge with the wild-type *B. pertussis*

strain. We found that mice immunised with the PagL-expressing strain, as compared to the mice immunised with the wild-type strain, had significantly lower lung CFU numbers at day 5 after the challenge, indicating that the vaccine based upon the PagL-expressing strain was more efficacious. This result shows, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

To identify immunological determinants that may be responsible for the increased vaccine potential of the PagL-expressing strain, we first analysed the *B. pertussis*-specific total serum IgG levels. In spite of the different vaccine efficacies observed, either of the wP vaccines induced comparable serum IgG titers. Protective mechanisms against *B. pertussis* have complementary roles for both cellular and humoral immunity (Mills *et al.*, 1998). With regard to the cellular immunity, protection against *B. pertussis* is correlated with Th1 responses, whereas with humoral immunity protection against *B. pertussis* is correlated with the production of IgG2a antibodies (Mills, 2001). As compared to the wild-type strain, immunisation with the both the PagP- and PagL-expressing strain induced higher *B. pertussis*-specific IgG2a titers. However, since the CFU numbers in the mice vaccinated with the PagP-expressing were not significantly lower than those in mice vaccinated with the wild-type strain, it cannot be concluded that the increased vaccine potential of the PagL-expressing strain is attributed to its potential to induce higher IgG2a antibody titers. Possibly, changes in antibody levels against a particular antigen play a role. Nevertheless, these results show that LPS composition influences the *B. pertussis*-specific IgG1/IgG2a ratio and thus plays a role in the Th1/Th2 balance after immunisation.

To gain more insight into potential differences in immune effector mechanisms induced by the different vaccines, we went further to analyse the post-challenge cytokine profile in the lung. For the majority of the eleven cytokines that we assayed for, we found no significant differences between the wP-vaccinated groups and the PBS/$AlPO_4$-treated control group. However, in the lungs of the mice immunised with the PagL-expressing strain, both the IL-1α and the IFN-γ levels were significantly higher. For IL-1α, this increase was only significant as compared to the PBS/$AlPO_4$-treated control group, but, for IFN-γ, it was significant as compared to all other groups. A role for IL-1α in the response to *B. pertussis* infection is not known, but its increased level suggests a stronger inflammatory response in the lungs after vaccination with the PagL-expressing strain. Studies in IFN-γ receptor-disrupted and IFN-γ knock-out mice have demonstrated that IFN-γ plays a key role in the protection against *B. pertussis* infection (Barbic *et al.*, 1997; Mahon *et al.*, 1997; Mills, 2001). Thus, the observation that immunisation with the PagL-expressing strain induced the highest levels of IFN-

γ in the lungs provides a possible explanation for the higher vaccine potential of this strain. Besides measuring the cytokine profile in the lungs, we have also measured the production of the same set of cytokines by splenocytes and cells from the bronchial lymph nodes after their stimulation *in vitro* with a *B. pertussis* whole-cell suspension or Concanavalin A, respectively. However, significant differences were not observed (data not shown).

To assess vaccine reactogenicity *in vivo*, we used two different approaches. In the first approach, which was aimed at determining transient effects on cytokine profiles directly after immunisation, we measured the concentration of two pro-inflammatory cytokines in serum samples taken 4 h after the primary and the booster immunisation. Consistent with the relatively high reactogenicity of wP vaccines, mice immunised with the wP vaccines exhibited high serum IL-6 levels as compared to the control group. However, the serum TNF-α levels in the wP-immunised groups were similar to those in the control group. This observation was unexpected since it has previously been reported that injection of LPS transiently increases both IL-6 and TNF-α levels in serum (Durand *et al.*, 2004). Possible explanations for this discrepancy are the nature of the LPS, i.e., *E. coli* LPS vs. *B. pertussis* LPS, the amount of LPS administered, the different genetic backgrounds, i.e., C57/BL6 mice vs. BALB/c mice, and the different route of administration, i.e., intramuscular vs. subcutaneous. Furthermore, Durand *et al.* (2004) showed that TNF-α levels, after reaching a peak concentration, decreased more rapidly than the serum IL-6 levels. Thus, possibly, the TNF-α levels had already returned to background levels 4 h after immunisation in our experiments. Nonetheless, since all three wP vaccines tested here induced similar increases in IL-6 levels, these vaccines appear to exhibit similar reactogenicity. In the second approach, we immunised mice with the different vaccines and measured their weight gain after 16 h. Consistent with the conclusion from the previous experiment, the results indicated that the reactogenicity exhibited by the wP vaccines was similar. However, 7 days after immunisation, mice immunised with the PagP-expressing strain exhibited significantly increased leukocyte numbers in their blood, which might indicate that the vaccine based upon the PagP-expressing strain contains a higher amount of active pertussis toxin than the other vaccines. Alternatively, because of its increased biological activity the PagP-modified LPS (Geurtsen *et al.*, 2006) might also contribute directly to leukocytosis by acting as B-cell mitogen.

The results presented here show that the expression of the LPS deacylase PagL increases vaccine efficacy without altering vaccine reactogenicity. Interestingly, this conclusion deviates from that of our previous study in which we showed that PagL-

expressing *B. pertussis* cells exhibit an increased toxicity *in vitro* (Geurtsen *et al.*, 2006). Thus, apparently, *in vitro* toxicity data cannot predict the outcomes of *in vivo* experiments, emphasising the importance of both *in vitro* and *in vivo* analyses. Since increased vaccine potency probably allows for a reduction of the vaccine dosage, the data presented here suggest that PagL-expressing *B. pertussis* strains may form a basis for the development of less reactogenic wP vaccines, as lower vaccine dosages will probably reduce vaccine side-effects. However, more detailed studies using different animal models, different *B. pertussis* strains, such as the vaccine strains, and vaccine dose-response studies should be performed. In conclusion, here we have provided the proof of principle that LPS-modifying enzymes are indeed promising tools for the development of safer wP vaccines. Obviously, this observation is not only significant in the field of pertussis vaccinology, but may also be applicable for the development of vaccines against other Gram-negative bacteria.

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