RESEARCH PAPER

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An improved whole cell pertussis vaccine with reduced content of endotoxin

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Abbreviations: wP, whole cell pertussis vaccine; aP, acellular pertusis vaccine; DTP, diphtheria-tetanus-whole cell pertussis combined vaccine; DTaP, diphtheria-tetanus-acellular pertussis combined vaccine; DTPlow, diphtheria-tetanus-pertussis low combined vaccine; LOS, lipo-oligosaccharide; LPS, lipopolysaccharide; Plow, pertussis low (whole cell pertussis vaccine with low endotoxicity); IB, Instituto Butantan (Brasil); NVI/RIVM, Netherlands Vaccine Institute/National Institute for Public Health and the Environment (The Netherlands); GC-LPS, capillary gas chromatography; LAL, limulus amoebocyte lysate assay; MWG, mouse weight gain test; HD, human dose; IOU, international opacity units; PT, pertussis toxin; ELISA, enzyme-linked immunosorbent assay; MPT, mouse potency test; FHA, filamentous hemagglutinin; Prn, Pertactin (69 kDa outer membrane protein); FACS, fluorescence activated cell sorter; dPT, detoxified pertussis toxin; MAT, monocyte activation test

An improved whole cell pertussis vaccine, designated as Plow, which is low in endotoxicity due to a chemical extraction of lipo-oligosaccharide (LOS) from the outer membrane, was evaluated for safety, immunogenicity and potency, comparatively to a traditional whole cell pertussis vaccine. Current whole cell pertussis vaccines are effective but contain large quantities of endotoxin and consequently display local and systemic adverse reactions after administration. Endotoxin is highly inflammatory and contributes considerably to the reactogenicity as well as the potency of these vaccines. In contrast, acellular pertussis vaccines hardly contain endotoxin and are significantly less reactogenic, but their elevated costs limit their global use, especially in developing countries. In this paper, bulk products of Plow and a traditional whole cell vaccine, formulated as plain monocomponents or combined with diphtheria and tetanus toxoids (DTPlow or DTP, respectively) were compared by in vitro and in vivo assays. Chemical extraction of LOS resulted in a significant decrease in endotoxin content (20%) and a striking decline in endotoxin related toxicity (up to 97%), depending on the used in vitro or in vivo test. The LOS extraction did not affect the integrity of the product and, more importantly, did not affect the potency and/or stability of DTPlow. Moreover, hardly any differences in antibody and T-cell responses were observed. The development of Plow is a significant improvement regarding the endotoxicity of whole cell pertussis vaccines and therefore a promising and affordable alternative to currently available whole cell or acellular pertussis vaccines for developing countries.

Introduction

Whooping cough or pertussis is an infectious disease of the upper respiratory tract caused by the Gram-negative coccobacillus *Bordetella pertussis* and a major cause of childhood morbidity and mortality. Pertussis is therefore still one of the world's leading causes of vaccine-preventable deaths. Worldwide 16 million cases of pertussis, 95% in developing countries, are registered every year, resulting approximately 195,000 deaths, mostly those under 6 mo of age.[1] Current pertussis vaccines mainly prevent disease and have limited impact on the circulation of the *B. pertussis*, even in countries with high vaccination coverage.[2] Vaccine or infection induced pertussis immunity wanes rather rapidly in time and individuals with feeble immunity may serve as reservoirs of infection for infants too young to be fully immunized.[2-7]

Whole cell pertussis vaccines (wP) developed in the 1940s were widely accepted despite the occurrence of local and systemic adverse reactions following immunization. However, perceived safety issues led to the development of acellular pertusis (aP) vaccines.[8] These vaccines were introduced in the late 1980s and nowadays are widely used in industrialized countries. A study conducted during the replacement of wP by aP in the Netherlands confirmed the lower reactogenicity of aP vaccines.[9] wP related adverse reactions are a hindrance for their continued

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Table 1. Quantitative analysis of LOS content and activity of 15 batches of Plow and bulk products in the GC and LAL assay, respectively

	Plow	wP	p (t-Test)
LOS content (μg LOS/mg protein)	5.27 ± 0.88	6.57 ± 0.87	**< 0.001***
Proportion of O-linked fatty acyl chains (C10:0–3OH/C14:0–3OH/C14:0)	0.9/2.2/1.0	0.9/2.2/1.0	
Endotoxin activity (IU endotoxin/mg protein)	**15,380 ± 13,851**	**533,317 ± 259,915**	**< 0.001****

*t-Test assuming equal variances; **t-Test assuming unequal variances.

use worldwide and they were therefore gradually replaced by aP in the industrialized world. However, aP are expensive and constitute an economic burden to developing countries.[10] The cost-benefit of replacing DTP (US$ 0.15 per dose) by DTaP (US$ 54.00 per dose) is therefore not justifiable for countries like Brazil.

Instituto Butantan (IB) in São Paulo, Brazil produces DTP vaccines for mass vaccination since 1983. Currently, more than 90% of the Brazilian children are vaccinated at the age of 2–4-6 and 18 mo of life, representing about 250 million doses administered, annually. A double-blind field study conducted in infants to compare two combined DTP-*Hemophilus influenza* type b vaccines, one of them entirely produced in Brazil and the other produced by GlaxoSmithKline,[11] showed no statistical differences regarding to immunogenicity, safety and consistency of production. In both groups, no severe adverse reactions directly related to the vaccines were observed. Moderate adverse reactions such as fever (around 85% after the first dose and 70% after the second), erythema, edema and heating (around 30%) and mild fever (in about 50%), however, were observed frequently. One of the most important factors that contribute to the reactogenicity of wP is the presence of lipo-oligosaccharide (LOS), the endotoxin from the bacterial outer membrane. Detoxification procedures are crucial steps during the production of wP. The endotoxin can be genetically or chemically detoxified and/or extracted from the outer membrane. On the other hand, LOS is a potent adjuvant of the immune system and changes in LOS composition or concentration could affect the vaccine-induced immune response.[12] To date, however, no pertussis vaccine containing genetically detoxified components is licensed. Instituto Butantan developed an improved wP vaccine that is low in endotoxicity by chemical extraction of LOS from the outer membrane (Plow).[13]

Results

Bulk products of plain wP and Plow, as well as diphtheria-tetanus-pertussis combined vaccines (DTP and DTPlow, respectively) were evaluated for safety, immunogenicity and potency in various in vitro and in vivo assays to substantiate the observed decline in endotxicity in the LAL assay after chemical extraction of LOS from the outer membrane of killed pertussis whole cells.

LOS- related toxicity. The LOS content (μg LOS/mg protein) in 15 batches of wP and Plow vaccines was determined by means of capillary gas chromatography (LPS-GC assay), and the endotoxicity by means of *Limulus Amoebocyte* Lysate (LAL) clotting assay (**Table 1**). The average LOS content of Plow and wP were determined at 5.27 and 6.57 μg LOS/mg protein, respectively. The mean in vitro endotoxin activity of Plow and wP were assessed at $1.54*10^4$ and $0.53*10^6$ IU endotoxin/mg protein, respectively. The chemical extraction of LOS from the outer membrane of pertussis whole cells reduced the endotoxin content in 19.8% ($p < 0.001$) without changing the proportion of the O-linked fatty-acyl chains in the Lipid A of the LOS (**Table 1**). The endotoxicity (LAL) of Plow was significantly reduced in 97% compared with the endotoxicity of wP products ($p < 0.001$; **Table 1**).

The discrepancy between the reduction in concentration and activity of endotoxin may be due to the presence of free endotoxin in the products. Free endotoxin is regarded to be more toxic than membrane bound LOS. Both products were spinned down for 20 min, 3000 rpm at 4°C and the supernatants tested for free LOS in the GC assay. Plow preparations contained 0.44 ± 16 μg/ml and wP preparations 3.84 ± 0.57 μg/ml of free LOS (data not shown), demonstrating that Plow hardly contains free endotoxin.

Different batches of wP and Plow vaccines produced at IB (n = 4) and NVI (n = 5) were tested in weight gain of mice at 16, 72 and 168 h (**Fig. 1**). The reduction of weight gain after the administration of the vaccines was regarded as an indicator of LOS-toxicity.[14] The observed weight reduction in the animals immunized with Plow, 16 h after the injection, was significantly lower compared with the injected with wP. Plow immunized mice recovered from the vaccination by showing a weight gain within 72 h after vaccination, whereas mice immunized with wP retained a backlog in body weight. These results indicated a limited inflammatory response after administration of Plow compared with wP.

Induction of pro-inflammatory cytokine IL-6. The production of pro-inflammatory cytokine is regarded as a sensitive measurement for LPS related toxicity.[15] Swiss mice were immunized intraperitoneally (ip) with one human dose (HD) of DTP or DTPlow vaccine (0.5 ml; 28 IOU/ml). The production of pro-inflammatory cytokine IL-6 was measured in sera obtained from blood samples taken 4 and 6 h after immunization. Significantly lower level of IL-6 was elicited by the DTPlow compared with DTP (**Fig. 2**). The reduced IL-6 production corresponded well to the lower weight loss and the rapid recovery of the mice immunized with Plow in the modified MWG test (**Fig. 1**).

Pyrogenicity of Plow and wP. The pyrogenicity of Plow and wP was assessed in rabbits to demonstrate a reduction in LOS induced fever.[16] Body temperatures were monitored continuously before and after primary and secondary intramuscular immunization with one HD of Plow (0.5 ml; 28 IOU/ml) or wP (0.5 ml; 32 IOU/ml) using temperature data loggers implanted in the peritoneal cavity of the animals. The circadian body temperature before immunization was used as an internal control of the normal body temperature of each individual rabbit. The changes

in temperature per rabbit and per immunization were calculated as the sum of the body temperature after immunization minus the normal circadian body temperature before immunization at each time point. wP showed an expected rise in body temperature after the first injection (**Fig. 3**), which was even higher after the second immunization. The total amount of the temperature increasing (Δ temperature area) for wP was 5.56 ± 0.30 and 8.76 ± 0.29 with a maximum increase in body temperature of 0.89°C ± 0.17 and 1.11°C ± 0.17 after the first and second immunization, respectively. Plow induced a significant lower increase in body temperature after both immunizations, confirming the reduction of endotoxicity observed in the other toxicity tests (**Fig. 3**). For Plow the mean Δ temperature area was 3.06 ± 0.21 and 3.39 ± 0.17 with a maximum increase in body temperature of 0.75°C ± 0.30 and 0.64°C ± 0.17 after the first and second immunization, respectively. In contrast to wP, Plow did not induce an elevated body temperature after the second immunization, demonstrating that Plow is better tolerated.

Morphological changes of B. pertussis cells in wP and Plow. Comparative analysis of *B. pertussis* cells before and after LOS extraction showed no difference in bacterial size and shape (**Fig. 4**). However, the outer cell membrane of the bacteria treated with the organic solvent appeared to be thinner. A similar effect was observed by other authors in a live genetically attenuated *B. pertussis* strain.[17] In our study, the outer membrane appeared as a shadow, which may be explained by assuming an increase in membrane fluidity caused by butanol within the hydrophobic layer of the membrane.[18]

Immunogenicity and potency. We have tested five plain batches of wP and Plow, as well as four final lots of DTPlow in the MPT and mouse immunogenicity test. After production, Plow and wP had similar potencies of 7.1 IU and 5.5 IU, respectively (p = 0.896; **Table 2A**) demonstrating that the LOS extraction had no influence on the potency of the vaccine. Additionally, the potency of different sets of Plow and DTPlow batches were also assessed after the production and after 3 y of storage at 4°C to determine whether their stability could be affected by the storage. Plow retained the potency assessed after production and after 3 y at 4°C when evaluated as a single component (potencies of 12.2 IU and 5.7 IU respectively; p = 0.837), and also when combined with diphtheria and tetanus toxoids (DTPlow) (potencies of 12.7 and 10.3, respectively; p = 0.655) (**Table 2B**).

Antigen specific antibody as well as T-cell responses induced in mice by wP and Plow were determined to substantiate the observed similarity in potency of both products. Swiss female adult mice (18–20 g) were immunized twice with one HD of DTP or DTPlow (0.5 ml; 28 IOU/ml; *ip*) at day 0 and 15. Specific IgG titers against pertussis major protective antigens (PT, FHA and Prn) were measured by ELISA in sera obtained 14 d after the second immunization (**Fig. 5**). The anti-PT and anti-Prn antibody titers of mice immunized with DTPlow were comparable to mice immunized with DTP (p = 0.09 and 0.06, respectively). Anti FHA antibody titers elicit by DTPlow in immunized mice were marginally lower (p = 0.036) than the induced by DTP.

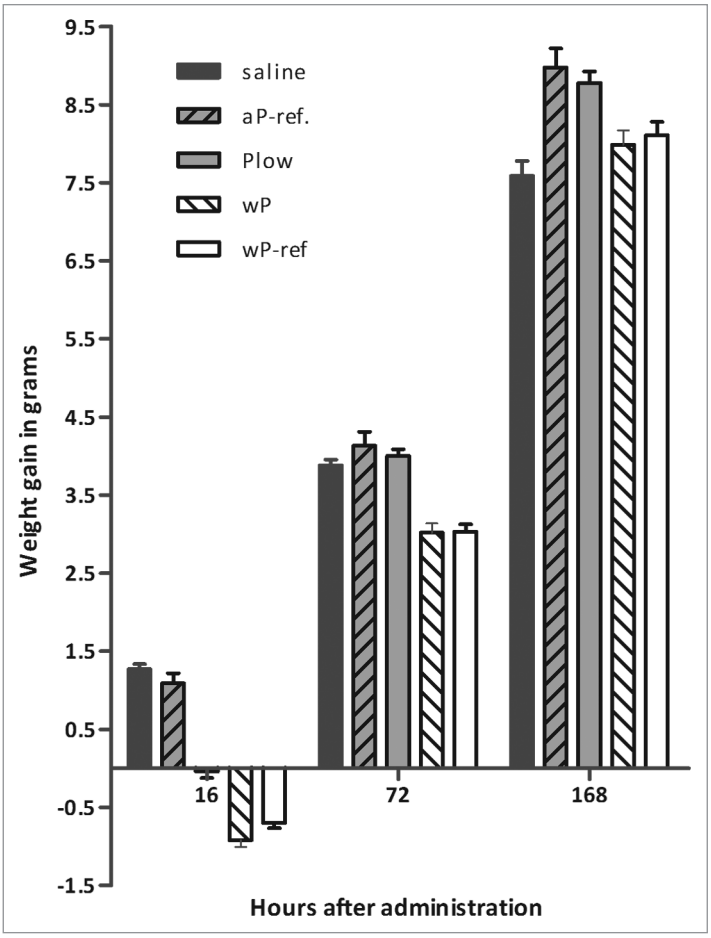

Figure 1. Mouse weight gain at 16, 72 and 168 h after administration of Plow and wP was used as indicator for LOS-toxicity. Reference wP and aP, produced at the RIVM, were used as high and low toxic references to illustrate the upper and lower limits in toxicity.

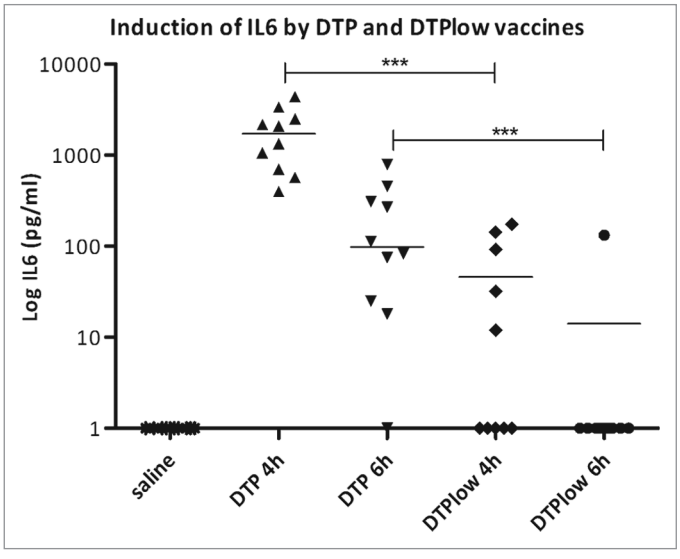

Figure 2. Induction of pro-inflammatory cytokine IL-6, 4h and 6h after primary immunization of mice with DTP or DTPlow (individual values of each animal; 10 animals per group). ***p ≤ 0.005.

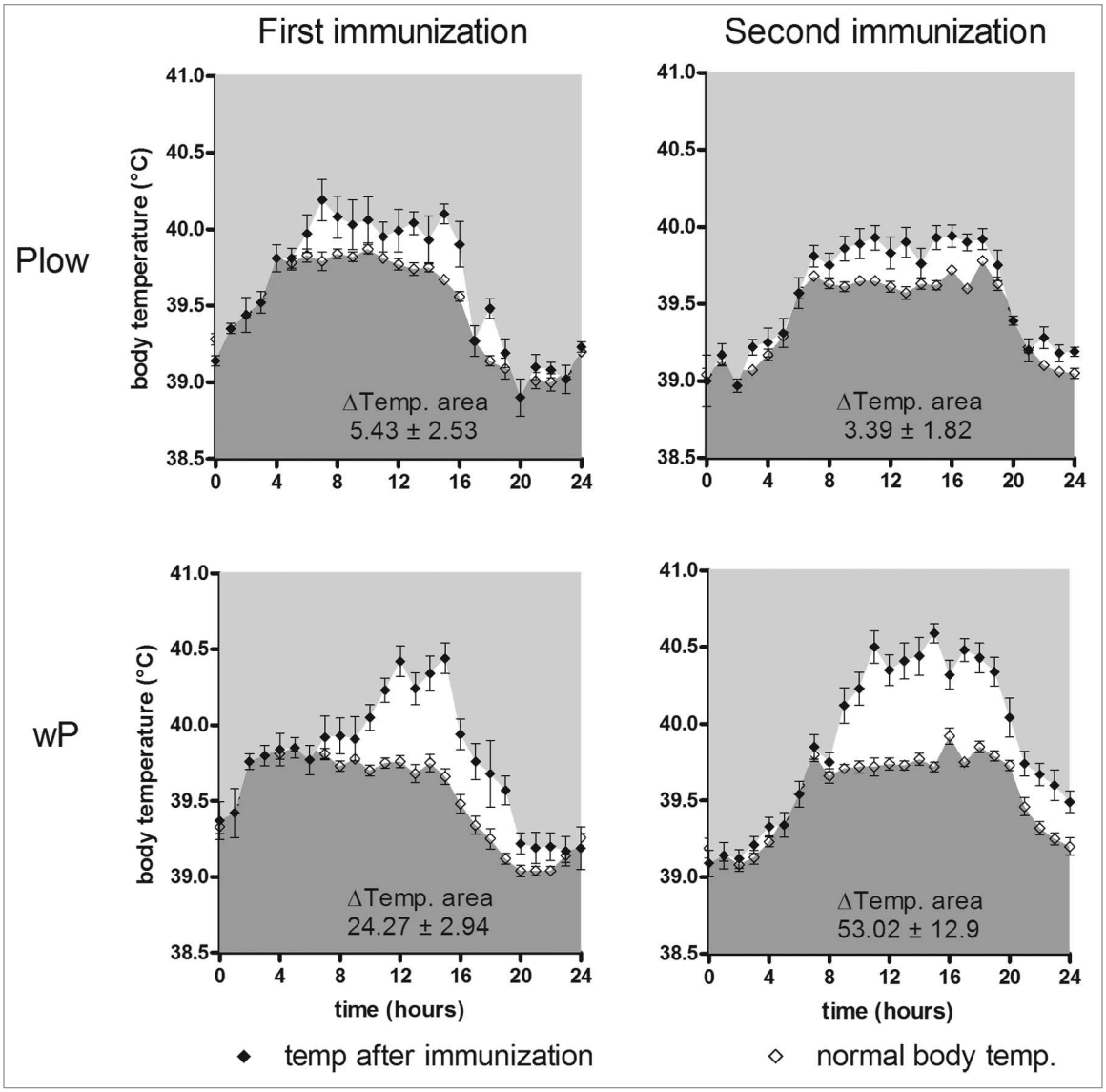

Figure 3. Body temperature measured continuously with implanted temperature loggers in rabbits (n = 4), before and after two immunizations with Plow and wP. The white area is the difference in normal circadian body temperature before immunization and increased body temperatures after immunization.

The thymus-dependent immunocompetence of the immunized animals was evaluated by flowcytometry, as the expression of CD4 and CD8 markers by splenocyte T cells,[19] after in vitro stimulation with detoxified pertussis toxin (dPT) (**Fig. 6**). The percentage of $CD4^+$ and $CD8^+$ T-cells in mice immunized with DTP or DTPlow was similar, showing intense proliferation of $CD4^+$ and very low levels of $CD8^+$ T-cells after stimulus with dPT. Measurement of IgG1/IgG2a subclasses against pertussis whole cells confirmed the similar Th1/Th2 responses elicited by DTPlow as well as DTP (**Fig.** 7), confirming that the LOS extraction had no effect on the immunogenicity of the pertussis antigens. Additionally, were also measured the potencies of the D and T components of DTP and DTPlow, by means of Toxin Binding assays.[20,21] Both preparations induced high and comparable levels of neutralizing antibodies against tetanus toxin (DTP 23.8 IU/ml and DTPlow 46.4 IU/ml; p = 0.27) and diphtheria toxin (DTP 66 IU/ml and DTPlow 32 IU/ml; p = 0.12), demonstrating that the LOS extraction had no effect on the immunogenicity against T and D antigens induced by the combined vaccine (data not shown).

Discussion

A traditional wP vaccine is produced for more than 30 y by Instituto Butantan. Thirty million doses of this vaccine, combined as DTP, are administrated to Brazilian children every year, free of charge, with over 95% of coverage, without major adverse events. Nevertheless, although rare, there is a risk of side effects related to wP and this risk remains a hindrance for its continued use worldwide. Traditionally produced wP contain large

quantities of endotoxin, which is a major factor contributing to local and systemic adverse events in humans.[9] A straightforward approach to limit the reactogenicity of these vaccines is to reduce their endotoxicity. The conventional wP detoxification procedures require a fastidious exposure of the cell suspension to formaldehyde, in order to obtain a decrease in toxicity. According to the methodology described by our group,[13,22] a chemical treatment with a mixture of water and butanol, in a short overnight period, partially extracts the LOS from the *B. pertussis* outer membrane.

LOS is considered as a crucial component of wP vaccine,[15] due to its adjuvant properties. A reduction of LOS may therefore affect the immunogenicity and potency of the Plow as well as the response to tetanus and diphtheria vaccine components in DTPlow. In our study, IB and NVI have independently evaluated wP and Plow products, in order to substantiate the significance of the LOS-related reduced toxicity in in vitro and in vivo toxicity tests, as well as, immunogenicity and potency of Plow and DTPlow comparatively to wP and DTP.

The lipid A part of LOS is mainly responsible for the endotoxicity and it is known that changes in its physicochemical properties may affect the biological activity.[15,23] In this work a distinct decrease in LOS related toxicity of Plow bulk products was consistently observed in all in vitro and in vivo toxicity assays. However, this reduction in the reactogenicity can only be partly attributed to the reduction of membrane bound LOS concentration after extraction. In addition, no significant differences in the physicochemical properties of the lipid A were observed. The distribution of all 5 fatty acids (GC-assay) are similar in wP and Plow products. Moreover, mass spectrometry of lipid A in both products showed no clear differences in the composition of LOS. Both products contained mainly mono- and di-phosphate penta-acylated lipid A within the normally observed heterogenicity of pertussis (data not shown).[15] To gain more insight into the reduction in endotoxicity, Plow and wP vaccines were tested in mice by a modified MWG-test.[24] Endotoxin related toxicity was clearly reduced after Plow administration, and the mice recovered more promptly from weight loss after injection with Plow as compared with wP, indicating a less severe toxic effect (**Fig. 1**).

The toxic and adjuvant activity of pertussis LOS are properties based both upon the recognition of the LOS by the host innate immune system and regulated by the production of pro-inflammatory cytokines e.g., IL-6 and the anti-inflammatory cytokines of immune cells.[25,26] The production of the pro-inflammatory cytokine IL-6 is regarded as a sensitive parameter to measure several responses to pyrogens and can be used as a measure of wP vaccines toxicity.[23] Recently, the in vitro monocyte activation test (MAT) has been accepted as an alternative to in vivo pyrogenicity.[27] The preferred readout of the MAT is usually IL-6, because unlike IL-1β and TNFα, IL-6 is secreted entirely into the cell-conditioned medium in large quantities, thereby permitting its complete estimation. However, IL-6 production was surprisingly high after stimulation with genetically detoxified wP preparations, while purified genetically detoxified LOS demonstrated a clear reduction in IL-6 production.[15] We therefore decided to evaluate the vaccine induced IL-6 production in mice. A significant reduction of reactogenicity was observed for DTPlow compared with DTP (**Fig. 2**) that corresponded well with the observed weight gain of the animals immunized with Plow (**Fig. 1**).

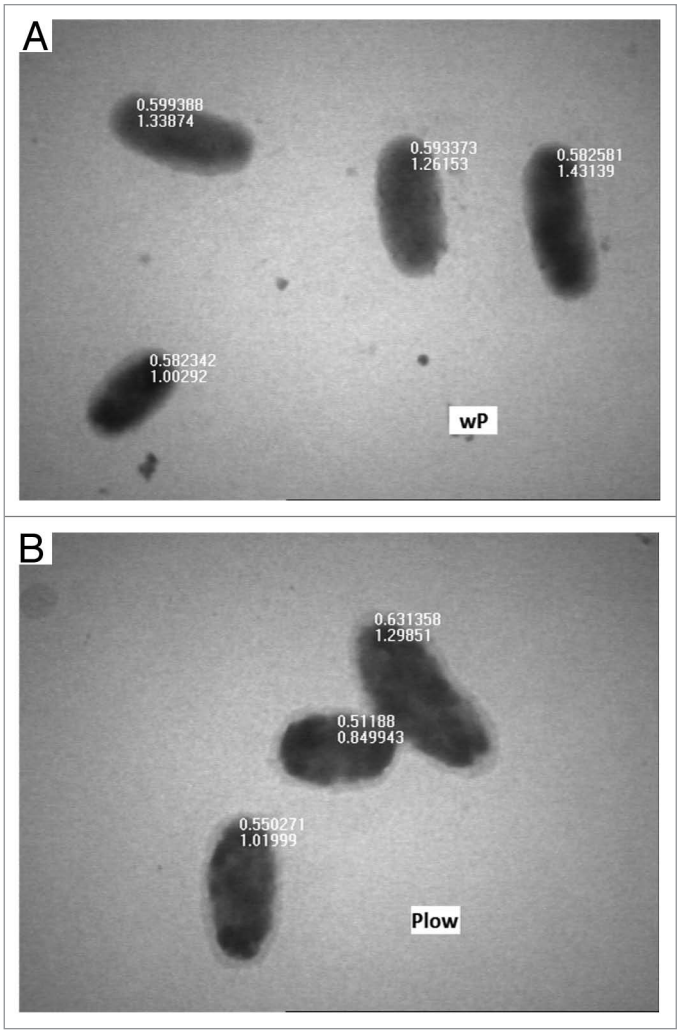

Figure 4. Electron microscopy of negatively stained whole cell pertussis vaccines: (**A**) before solvent treatment (wP) and (**B**) after solvent treatment (Plow). The dimensions of the cells are indicated in micrometers.

Free endotoxin is generally much more toxic than membrane bound LPS[28] and the content of free LOS content in wP seems to determine the degree of LOS related toxicity.[23] The difference in reduction of the actual content of LOS (20%) and its biological activity (97%) of Plow compared with wP is most likely cause by the amount of free LOS in both products. The supernatants of Plow and wP products were tested for free LOS and the concentration of free LOS in the supernatant of the wP was almost 10 times higher than in Plow. This reduction corresponds well to the reduction of endotoxicity measured in the LAL assay, as well as, the reduction in IL-6 production in mice. LOS-extraction from the outer membrane of pertussis whole cells reduced not only the amount of membrane-bound LOS (± 20%) (**Table 1**) but also the amount of free LOS (± 90%) in Plow products resulting in a striking reduction of endotoxicity (97%), by the LAL assay

Table 2. Potencies of Plow, wP, DTPlow and DTP bulk products estimated by means of the MPT

(A) Potency of IB pertussis bulk products after production							
	batch	**02/05**	**03/05**	**04/05**	**01/06**	**04/06**	**wgm**
Plow (32 IOU/ml)	Potency (IU)	9.6	4.8	8.0	7.9	8.1	7.1
wP (32 IOU/ml)	**batch**	**31/05**	**34/05**	**36/05**	**02/06**	**44/06**	**wgm**
	Potency (IU)	5.6	7.3	4.5	7.3	4.2	5.5
Chi 2 test#	p = 0.896; proportion Plow/wP = 1.26 (0.56 - 2.84)						
(B) Product stability after 3 y of storage at 4°C							
Plow (32 IOU/ml)	**batch**	**05/05**	**06/05**	**01/06**	**03/05**		**wgm**
assessed after production	Potency (IU)	13.1	12.2	15.6	9.6		12.2
assessed after 3 y	Potency (IU)	4.0	4.8	6.6	6.8		5.7
Chi 2 test#	p = 0.837; proportion NVI/IB = 0.46 (0.23 - 0.94)						
DTPlow (28 IOU/ml)	**batch**	**03/05**	**01/06**	**03/06**	**01/05**		**wgm**
assessed after production	Potency (IU)	11.7	21.3	12.3	10.6		12.7
assessed after 3 y	Potency (IU)	8.8	6.4	11.9	14.7		10.3
Chi 2 test#	p = 0.665; proportion NVI/IB = 0.84 (0.40 - 1.74)						

#Chi-square test of homogenicity: potencies analyzed for similarity after log transformation and using the confidence intervals as weighing factor of the test reliability. Wgm, weighed geometric mean.

(**Table 1**). The significant reduction in free LOS may also be the main cause of the Plow induced limited inflammatory response and consequently the reduced fever after vaccination (**Fig. 3**). Apparently, the chemical extraction of LOS has an additional effect on the release of LOS from the outer membrane into the solvents and may prevent or dramatically slow down this release. In traditionally produced wP, 35–60% of the LOS is released in the solvents during the first week and up to 80% after 5–6 mo of storage at 4°C.[29] On the other hand, the chemical extraction of LOS did not substantially interfere with the integrity of the pertussis whole cells, since their morphological characteristics were maintained after the solvent treatment, as observed by electron microscopy (**Fig. 4**).

The reduced concentration and activity of endotoxin in Plow and DTPlow bulks does not affect the potency of both products, which seems to be highly stabile (**Table 2**). However, a real time stability study for toxicity as well as potency with Plow and DTPlow should demonstrate whether the release of LOS is stopped permanently or increases in time.

Although little is known about the protective immune mechanisms in the MPT, antibodies as well as cellular immunity are required for protection against disease.[25] wP induces a balanced Th1/Th2 response similar to natural infection and changes in T-cell responses may affect the effectiveness of the vaccine induced immunity.[19] Higgins and colleagues demonstrated that wP activates innate immune cells through TLR4.[30] TLR4 helps to direct the induction of Th1 and Th-17 cells, which mediate protective cellular immunity to *B. pertussis*. While the reactogenicity of LPS is an impediment for its use as an adjuvant, this same characteristic have led some authors to consider the LPS as a crucial component of the whole cell pertussis vaccines in mice.[31] In our study, the evaluation of Plow and wP vaccines in a series of in vitro and in vivo assays demonstrated that the chemical extraction of LOS results in reduced LOS-related toxicity without affecting the immunogenicity or potency of Plow products. We have demonstrated that the LOS-extraction has little or no effect on non-LOS related product properties. In fact, the substitution of wP for Plow did not interfere in the vaccine induced antibody responses against pertussis antigens (**Fig. 5**), nor has it changed the IgG1/IgG2a subclass distribution (**Fig. 7**) or dPT specific T-cell responses (**Fig. 6**). Similar to DTP, the combined DTPlow vaccine also elicited high titers of neutralizing IgG antibodies against tetanus and diphtheria toxins, but a real time stability study of the potency should warrant the observation that LOS-extraction does not interfere with the stability of the product. As a whole, these results indicate that the adjuvant activity of LOS on the P, D and T vaccine components are not affected by the chemical reduction of LOS in DTPlow.

These observations are substantiated by results of a Phase I trial with wP and Plow in Brazil.[32] In this study, 115 infants were immunized with DTPlow and 119 with DTP. No severe local or systemic adverse events were noted after vaccination with both vaccines. The IgG antibodies titers against the DTP components and the frequencies of proliferating $CD4^+$ and $CD8^+$ T cells were similar for both groups, demonstrating that the LOS-extraction did not affect the vaccine induced immunity in infants. However, although the consistently observed reduction in LOS related toxicity of Plow bulk products in all in vitro and in vivo toxicity assays, with no interference in the immunogenicity or potency of the vaccine, in preclinical trials, we cannot conclude that this new vaccine is less reactogenic in humans, since it was not detected severe reactogenicity induced by both, Plow or the traditional wP vaccine. The results in preclinical assays make us infer a lower reactogenicity in humans, but this would be only possible evaluating a higher sample of newborns or in a clinical trial in adolescents and young adults, where the Plow would be considered as a booster dose, a good alternative for developing countries which cannot introduce the reinforcing of pertussis in adults due to the high cost of the aP vaccines.

The development of DTPlow can be a significant improvement regarding the endotoxicity of wP and a promising and affordable

alternative to the currently available pertussis vaccines, especially for the developing world. Furthermore, its production process does not alter the cost of the traditional DTP and also provides a by-product, a raw material for obtaining the monophosphoryl-lipid A (MPLA),[33] which can be used as adjuvant for other vaccines and in fact, was successfully coadministered with influenza vaccine.[34] Our data strongly suggest a potential additional safety of whole cell pertussis vaccines, with possible cost-benefits gains in its production process.

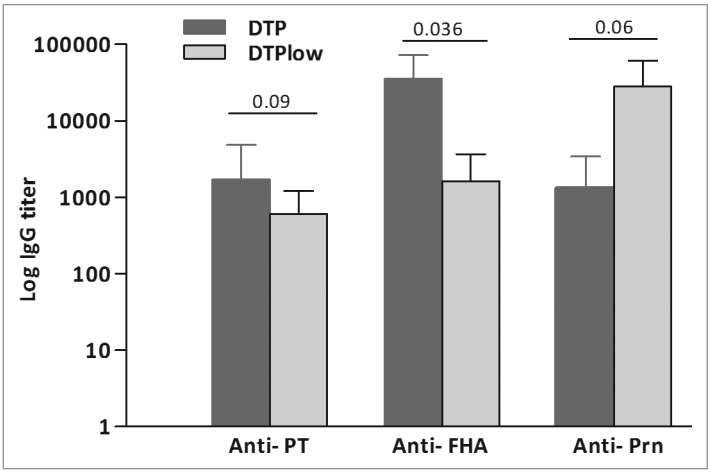

Figure 5. IgG anti-pertussis toxin (PT), anti-filamentous hemagglutinin (FHA) and anti-pertactin (69 K) antibodies (log titer) elicited in mice immunized with DTP or DTPlow vaccines. Significant p-values < 0.05 (Mann Whitney test).

Materials and Methods

Production of wP and plow. *B. pertussis* strain 137 was cultured under the same conditions utilized in the production of wP by IB. Briefly, the *Bordetella pertussis* working seed lot was reconstituted in modified Stainer and Scholte medium and grown in modified Bordet-Gengou medium supplemented with sheep blood, to obtain a pre-fermentation culture to be utilized as inoculum in a 750 or 1,000 L fermentation unit. The product was inactivated with formaldehyde solution and concentrated in a saline solution under a tangential filtration system, resulting in wP. Plow was prepared by treatment of wP with a mixture of butanol and water which was subsequently removed entirely by centrifugation, washing steps and tangential filtration. wP and Plow plain vaccines were also produced at lab scale at the former Netherlands Vaccine Institute (NVI), The Netherlands, using the IB protocol with two exceptions. At the NVI, *B. pertussis* strains B134 and B509 were cultured in a defined synthetic medium[35] and wP bulk products were heat inactivated in the presence of 16 mM formaldehyde.

Quantitative analysis of the fatty acids in pertussis LPS. The analysis of fatty acids was performed by capillary gas chromatography (GC-LPS), using GC equipped with a Flame Ionization detector.[36] The GC method includes the following treatment for both standards and samples. First a hydrolysis step is performed to separate the fatty acids from the lipid-A part of the LPS. Saponification reagent (3.75 M NaOH I 50/50 v/v methanol/water) is added to the pertussis biomass and incubated for 30 min at 100°C. This means that both the N-bound fatty acids and the O-bound fatty acids are released. The next step is the methylation of the released fatty acids under acidic conditions. HCl (3.25 M) in methanol is added to the samples and incubated for 20 min at 80°C. Finally, after an extraction step by which the methylated fatty acids are dissolved in an organic phase (hexane-MTBE), the material is injected into an Agilent 109091B-102 capillary column of the GC (6890 Agilent gas chromatograph) and the concentrations of pertussis LOS specific fatty acids C10:3OH and C14:3OH next to the non-specifc C:14 are detemined. Fatty acids (SIGMA) are quantified against calibration curve of diluted solutions of pure fatty acids in dichloromethane in the presence of an internal standard. The fatty acid levels (expressed in μmol/ml) are estimated by linear regression using standard Agilent software package and their concentrations correspond to LPS-concentration. Prior to quantification, a calibration mix (Microbial ID Inc. USA) of methylated fatty acids is analyzed to determine the exact retention times of all relevant methylated fatty acids.

Lipid A analysis. LOS was isolated by an isolation of lipid A from bacterial cells with sequential release of their ester-linked fatty acids by a mild alkali treatment.[37] A structural analysis of lipid A is performed by nanoelectrospray tandem mass spectrometry on a Finnigan LCQ in the positive ion mode as described previously.

Limulus amoebocyte lysate assay (LAL). Batches of wP and Plow vaccines were assayed for endotoxin content[38] with the chromogenic LAL test at IB and with a gel clotting assay at the NVI, in parallel with a control standard endotoxin, according to the manufacturer's instructions [Cambrex –Limulus Amebocyte Lysate (LAL) Pyrogent® Plus].

Mouse weight gain test (MWG). At IB, Swiss female mice (18–20 g, 10 animals per group) were intraperitoneally (*ip*) injected with about a human dose (HD) of the vaccine samples [0.5 ml, containing 32 or 28 international opacity units (IOU)/ml of wP or Plow, respectively] or saline as a negative control. The vaccine passed the test if: (1) at the end of 72 h, the total weight of the group was not lower than the initial weight; (2) at the end of the 7th day, the mean weight gain of the group was not lower than 60% of the mean weight gain of the negative control group and (3) more than 95% of the animals inoculated with the sample survived.

At the NVI, a modified MWG was performed.[24] Briefly, five or 10 RivmN/NIH male mice (14 – 17 g) per group were injected *ip* with half a human dose of wP or Plow (0.5 ml, 16 or 14 IOU/ml, respectively). Mice were weighed individually at 16 h, 3 and 7 d after injection. The number of white blood cells was determined at day 7, as a parameter for Pertussis Toxin (PT) induced toxicity.

IL-6 induction assay. Groups of 6 outbred Swiss female adult mice (18–20 g) were immunized *ip* with a HD of DTP or DTPlow (0.5 ml containing 32 or 28 IOU/ml, respectively). The mice were bled at 4 and 6 h after the first immunization. IL-6

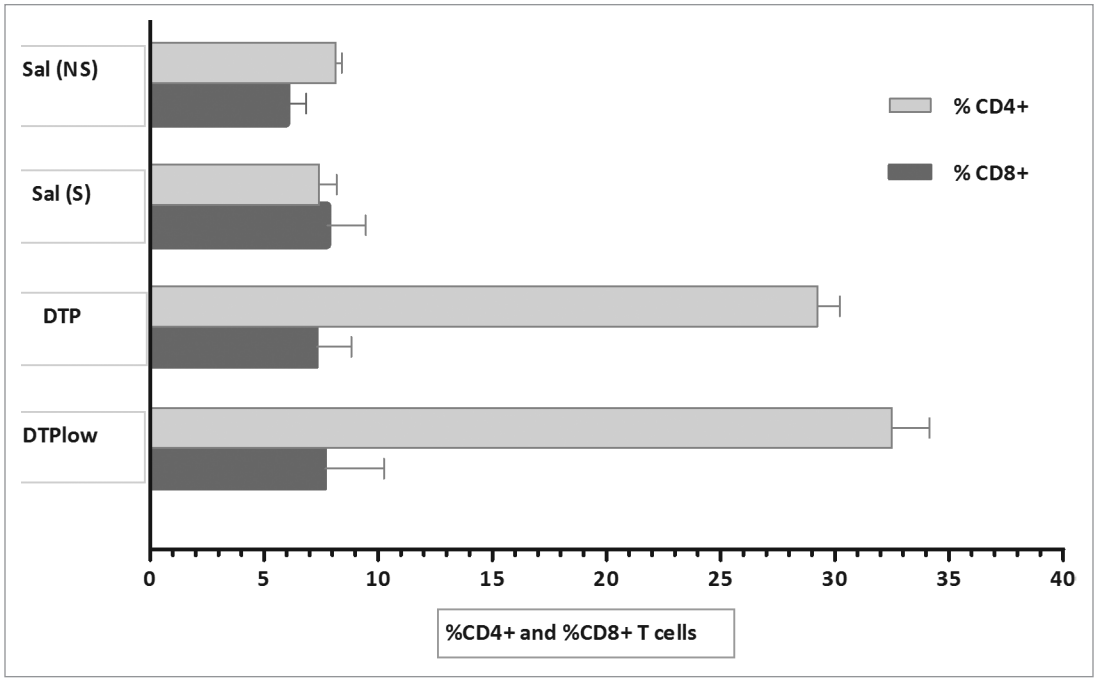

Figure 6. CD4+ and CD8+ T cell subsets (%) in spleen cells of mice immunized with DTP or DTPlow. The cells were in vitro stimulated with detoxified pertussis toxin (dPT). The control groups are composed of spleen cells obtained from mice injected with saline (Sal) and stimulated (S) or not stimulated (NS) with dPT.

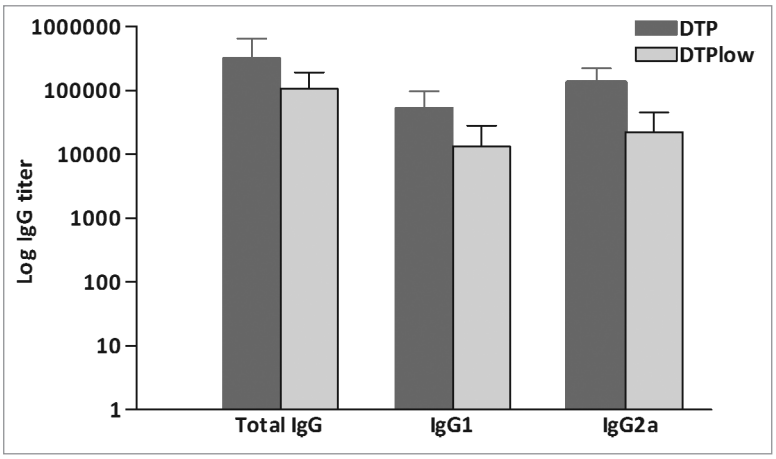

Figure 7. Total IgG, IgG1 and IgG2a anti-wP log antibody titers in mice immunized with DTP or DTPlow.

concentrations in the sera were quantified by an enzyme-linked immunosorbent assay (ELISA) for mouse IL-6 according to the manufacturer's instructions (Peprotech Inc.).

Vaccine-induced fever in rabbits. Body temperature after administration of Plow or wP was monitored continuously in handled female HsdRIVM/Elco rabbits by means of temperature data loggers (DST micro-T, Star-Oddi) according to NVI/RIVM protocol.[16,39] Four rabbits (2–3 kg) per vaccine were injected intramuscularly with one HD of Plow (0.5 ml; 28 IOU/ml) or wP (0.5 ml; 32 IOU/ml) and boosted with the same dose 4 weeks later. One week after the second immunization the rabbits were euthanized. Data were extracted from the implanted temperature loggers. The mean normal circadian body temperature of each individual rabbit during a period of 7 d before the first and second immunization was calculated and subtracted from the circadian body temperature during the first 24 h after the first and second immunization, respectively, demonstrating the vaccine induced increase in body temperature.

Mouse potency test (MPT). Potency of wP and Plow were evaluated by means of the MPT at IB and NVI.[40] The potency of each bulk products was estimated in International Units (IU), by comparison with the obtained from an "in house" reference vaccine, previously calibrated against an International Standard for Pertussis Vaccine, the National Institute for Biological Standards and Control (NIBSC). Female Swiss or RivmN/NIH mice (14–16 g) were immunized *ip* with a 5-fold dilution series of wP or Plow (0.5 ml/dilution, 16 animals per group). After 14 d, the animals were intracerebrally challenged with a virulent strain of *Bordetella pertussis* ATCC 18323 (0.03 ml/dose of a bacterial suspension of 10 IOU/ml diluted 1/3,000). The number of survivors was monitored up to 14 d after challenge and the level of protection was expressed as the percentage of live animals. The deaths up to 72 h after the challenge were attributed to manipulation errors and were excluded from the test. It was considered that the vaccine passed the recommendations when its estimated potency was not less than 4.0 IU in the volume recommended for a single human dose and if the lower fiducial limit (p = 0.95) of the estimated potency was not less than 2.0 IU.[40]

Humoral immune response induced by DTP and DTPlow. Groups of 6 outbred Swiss female adult mice (18–20 g) were immunized with DTP or DTPlow (0.5 ml containing 32 or 28 IOU/ml, respectively, previously adsorbed with aluminum hydroxide 1.2 mg/ml). A booster was given under the same conditions 14 d after the first injection. Serum samples were collected by retro-orbital puncture, 14 d after the second immunization. Control groups were constituted of mice injected with saline solution or with the adjuvant. Total IgG was measured by a modified ELISA,[41] in individual sera, against purified pertussis toxin (PT; 1 μg/ml; Sigma); filamentous hemagglutinin (FHA; 2 μg/ml; FDA/CBER—USA), Pertactin (69 kDa outer membrane protein) (Prn; 4 μg/ml; kindly provided by Dr. Mariagracia Pizza, Novartis) and tetanus and diphtheria toxoids (5 Lf/mL, Instituto Butantan), in 50 mM carbonate/bicarbonate coating buffer, pH 9.6, overnight at 4°C. For anti-pertussis isotyping assays the sera were evaluated against wP vaccine suspension (approximately 10^9 cells/ml) and it was used goat anti-mouse IgG1 and IgG2a conjugates (Sigma). The antibody titers were defined as the reciprocal of the maximum serum dilution that resulted in $OD_{492nm} \geq 0.1$.

FACS analysis of T cell phenotypes. $CD4^+$ and $CD8^+$ T cells were evaluated by flow cytometry, under standard procedures, in splenocytes of mice immunized with DTP or DTPlow. Briefly, spleen cells were obtained from three mice per group, 16 d after immunization and cultured (2×10^6/ml) during 72 h following stimulation with detoxified PT (dPT) (1 μg/ml). The control group consisted of non-stimulated spleen cells obtained from mice injected with saline. Concanavalin A (5 mg/ml) was used as a positive control for cell reactivity. T cell phenotypes were analyzed using mAb's against murine CD8 (rat; anti-$CD8^+$-PE Phar-Mingen), and CD4 (rat; anti-$CD4^+$-FITC Phar-Mingen) T cells. Data were recorded by FACScan[(Fluorescence Activated Cell Sorter, FACSCalibur (Becton Dickinson)].

Morphological analysis of wP and Plow. wP and Plow morphology were examined under transmission electron microscopy, using a flotation negative staining procedure, as previously described,[42] with the following modifications: parlodium carbon coated copper grids were floated for 1 min onto a 20 μl-drop of a suspension with approximately 10^9 bacteria/ml. After 30 s air-drying, the grids were floated for 2 min onto a 20 μl-drop of 2% phosphotungstic acid (pH 7.0) stain and examined, after air-drying, in a transmission electron microscope (LEO906E) at 80 kV.

Ethics Committee for animal experimentation. The experiments using animals were previously approved by the Ethics Committee for experiments with animals of the Butantan Institute (Brasil) or the National Institute for Public Health and the Environment (RIVM) (The Netherlands).

Statistical analysis. The significance of differences in immunogenicity between the studied groups were analyzed using Mann Whitney test (significant p-values < 0.05) or χ^2-test of homogeneity for analysis of similarity in potency (significant p-values $0.05 \leq p \leq 1.0$).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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