Biological activities of lipoteichoic acid and peptidoglycan–teichoic acid of *Bacillus subtilis* 168 (Marburg)

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To evaluate the suitability of *Bacillus subtilis* as a production host of heterologous proteins for pharmaceutical purposes, we assessed the biological activity of this bacterium and its major cell envelope components, lipoteichoic acid (LTA) and peptidoglycan–teichoic acid complex (PG–TA) in several eukaryotic effector assays. LTA and PG–TA were found to be non-toxic for mice and guinea-pigs in a short-term toxicity assay. PG–TA was weakly pyrogenic and weakly mitogenic. Both LTA and PG–TA acted as immunologic adjuvants in mice and when injected in mice, also caused an increase in the number of granulocyte–monocyte colony-forming cells in the bone marrow probably via stimulation of production of granulocyte–macrophage colony-stimulating factor.

Introduction

The production of heterologous proteins in *Bacillus subtilis* for vaccine purposes has been suggested for several reasons. This bacterium is widely used in industry (Debabov, 1982; Harwood, 1992) and efficient expression systems have been developed for the production of proteins either intracellularly or in secreted form (Sarvas *et al.*, 1990). *B. subtilis* is nonpathogenic and devoid of known toxic components, including endotoxin. We have previously described the use of *B. subtilis* as a host to produce several vaccine protein candidates, e.g. the subunits of pertussis toxin (Runeberg-Nyman *et al.*, 1987; Himanen *et al.*, 1990) and the class 1 outer membrane protein (P1) of *Neisseria meningitidis* (Nurminen *et al.*, 1992). The heterologous proteins could be purified to contain only small amounts (less than 1 %) of lipoteichoic acid (LTA) and peptidoglycan–teichoic acid complex (PG–TA), the most abundant of the many cell envelope components of *B. subtilis* (Himanen *et al.*, 1993).

In a protein vaccine, it is important to be aware of any additional components that may possess biological activities e.g. affecting the host's immune response. An evaluation of the expected effects of LTA and PG–TA in such a vaccine on the basis of the reported bioactivities of teichoic acids and PG is difficult because of their different sources and purification methods, and incomplete chemical characterization of the preparations. For this reason, we have now characterized the LTA and PG–TA of the *B. subtilis* production strain 168 (Marburg) both chemically and in respect of a number of bioactivities.

Methods

Bacterial strain and growth conditions. The *Bacillus subtilis* strain IH6564 (Himanen *et al.*, 1990) was derived from IH6140, a protease-deficient derivative of *B. subtilis* 168 (Marburg) (1A289 in the Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA). It was grown as described by Himanen *et al.* (1991). The cells were collected, washed with physiological saline and freeze-dried as indicated.

Purification of LTA and PG–TA. LTA was prepared according to Fischer *et al.* (1983). The bacterial cells in 100 mM-sodium citrate buffer (pH 3) were disrupted by vigorous blending in the presence of glass beads and extracted by 40 % (v/v) phenol, at pH 4.7, 65 °C. The aqueous phase was dialysed to remove phenol, treated with RNAase at 55 °C for 2 h, dialysed against 100 mM-sodium acetate (pH 4.7) and freeze-dried. LTA was further purified by FPLC on Octyl-Sepharose CL-4B (Pharmacia). Crude LTA was applied to the column (24 × 370 mm) equilibrated with 15 % (v/v) 1-propanol in 100 mM-sodium acetate (pH 4.7) and eluted with a linear gradient of 1-propanol (15–70 %) at a flow rate of 0.5 ml min⁻¹. LTA eluted as a distinct fatty-

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Abbreviations: ConA, Concanavalin A; DAP, *meso*-diaminopimelic acid; GM-CSF, granulocyte–macrophage colony-stimulating factor; LAL, *Limulus* amoebocyte lysate assay; LTA, lipoteichoic acid; PG–TA, peptidoglycan–teichoic acid complex; SRBC, sheep red blood cells.

acid-containing peak with 65% 1-propanol. The fatty-acid-containing fractions were pooled, dialysed against water and freeze-dried to yield 23 mg LTA (g dry wt bacteria)⁻¹.

PG-TA was prepared as described by Aono & Horikoshi (1983). The wet bacterial cells in 50 mM-Tris (pH 7.5) were disrupted in a French Pressure Cell in the presence of 5 mM-EDTA and 5 mM- β -mercaptoethanol. The broken cells were extracted with 4% (w/v) SDS (Fluka) at 100 °C for 30 min and subsequently at 37 °C overnight. The insoluble material was collected by centrifugation (17000 g, 20 min) and washed sequentially with 4% SDS, water, twice with 1 M-NaCl and twice with water before freeze-drying to obtain purified PG-TA. The yield was 250 mg (g dry wt bacteria)⁻¹.

Animals. The following animals were used as indicated in the text: clinically healthy outbred NIH/S mice, coloured guinea-pigs and Chinchilla-Bastard rabbits (all bred in a conventional environment in National Public Health Institute, Helsinki); inbred BALB/c mice (barrier bred in the Laboratory Animal Centre, University of Helsinki, Helsinki); inbred CBA/N mice and F₁-hybrid (CBA $\times$ C57BL) mice (bred in a conventional environment in 'Stolbovaya' nursery, Academy of Medical Sciences, Moscow).

Chemical methods. Proteins were measured by the Lowry method. A dot-blot method was used for semiquantitative estimation of DNA: 0.4 μ l of the samples were applied to a 0.9% agarose plate containing ethidium bromide, and the amount of DNA was estimated under UV light by comparing the intensities of the blots to those obtained with graded amounts of commercial DNA (Boehringer Mannheim). Sodium was determined using a Ciba Corning 614 Na⁺/K⁺ Analyzer. Phosphate was determined by the method of Lowry *et al.* (1954) and total hexosamine by the Morgan-Elson reaction after hydrolysis of the samples in 4 M-HCl at 100 °C for 16 h (Strominger *et al.*, 1959). Fatty acids were determined by GLC as their methyl esters after methanolysis using 2 M-HCl in MeOH for 7 h at 86 °C. Neutral sugars were liberated by acid hydrolysis (0.1 M-HCl, 100 °C, 48 h) and determined by GLC as their alditol acetates (Sawardeker *et al.*, 1967). Alanine and meso-diaminopimelic acid (DAP) were determined as their *N*-heptafluorobutyl isobutyl esters (Sonesson *et al.*, 1988). Reference DAP and *N*-heptafluorobutyric anhydride were obtained from Sigma. Glycerol was determined by HPLC after hydrolysis of the samples in 5.8 M-HCl at 100 °C for 75 h (Himanen, 1992). Instrumentation and conditions of GLC and combined GLC-MS were summarized recently (Helander *et al.*, 1992).

Test for acute toxicity. The test was performed according to the *European Pharmacopoeia* (Part 1, V.2.1.5, 2nd edn, 1986), for detecting abnormal toxicity in the last stage of the vaccine production process. Five male NIH/S mice (weighing 17–22 g) and two guinea-pigs (male or female, 250–350 g) were used for each preparation. The animals were injected intraperitoneally with the preparations followed by an observation period of 7 d. The animals were weighed before and at the end of the observation period.

Pyrogenicity. The pyrogenicity assay was carried out according to the *European Pharmacopoeia* (Part 1, V.2.1.4, 2nd edn, 1986). Rectal temperatures of the rabbits (males or females, 2.8–4.3 kg) were monitored for 90 min before and 3 h after intravenous injections of the preparations (1.0 ml kg⁻¹). Each sample was considered non-pyrogenic if the summed fever response of three rabbits did not exceed 1.15 °C, and pyrogenic if the response exceeded 2.65 °C.

Limulus amoebocyte lysate (LAL) assay. The LAL assay was performed according to Cooper & Neely (1980) using E-tect lysate (Farnos Diagnostica, Turku, Finland) with a label sensitivity of 0.014 ng ml⁻¹. Endotoxin from *Escherichia coli* O55:B5 (Whittaker M.A. Bioproducts) was used as a standard. The assay was performed as follows: diluted preparations and endotoxin standard were pipetted into tubes, E-tect lysate was added and the tubes were incubated at

37 °C for 60 min. If a clot that sustained inversion of the tube was formed, the preparation was considered to be LAL-positive. By comparison with the standard, the amount of LPS in the test sample could be expressed in ng ml⁻¹.

Mitogenic assay. Spleens of 5–8-week-old CBA/N mice (weight 18–20 g; killed by cervical dislocation) were removed aseptically, cells were teased out, washed and placed in RPMI 1640 medium (Flow Laboratories) supplemented with 5% (v/v) heat-inactivated foetal calf serum (Flow), 2 mM-L-glutamine (Flow), 50 μ g gentamicin ml⁻¹ (Flow) and 5 $\times$ 10⁻⁵ M-2-mercaptoethanol (Merck). Cultures containing 2.5 $\times$ 10⁵ viable cells were established in microplates. Appropriate concentrations of freeze-dried *B. subtilis* cells, LTA, PG-TA, LPS (*E. coli* O55:B1; Sigma) or Concanavalin A (ConA; Pharmacia) were added. Cultures were incubated at 37 °C, 5% (v/v) CO₂ and 100% humidity, and pulsed with [³H]thymidine [1 μ Ci (37 kBq) per well]. The cells were collected and radioactivity was determined.

Haematopoietic precursor cells. Male BALB/c mice (18–20 g) were injected intraperitoneally with the test preparations. To obtain the control level no injection was done. The mice were killed by cervical dislocation at different times, their femurs prepared, flushed with the medium and the number of cells in the suspension counted. The medium for the bone marrow cultures was Iscove (Flow) enriched with 10% foetal calf serum (Flow), 50 μ M-2-mercaptoethanol (Merck) and antibiotics (penicillin, streptomycin, and gentamicin; 50 μ g ml⁻¹). Difco Bacto-agar was solubilized in hot water, mixed with the bone marrow cells in complete medium and placed in a water bath at 37 °C. Cell suspensions in agar (0.3%) medium were dispensed into plastic culture plates to which a standard GM-CSF-containing material, a supernatant of MK-1 hybridoma cell cultures (kindly donated by Dr L. V. Carcanitsa, Institute of Haematology and Blood Transfusion, Minsk, Belorussia) had been added. The MK-1 hybridoma had been obtained by fusion of ConA-stimulated AKR mouse thymocytes with the syngeneic tumour cell line BW 5147; the conditioned medium of MK-1 cells stimulated the formation of granulocyte and macrophage colonies in semi-solid agar cultures of murine bone marrow cells in a species-specific manner, and there was no evidence of cytokines other than GM-CSF (Carcanitsa, 1988). The cultures were incubated for 7 d at 37 °C with 5% CO₂ and 100% humidity, and the number of colonies was counted. In *in vitro* experiments the preparations were added to a culture of normal BALB/c mouse bone marrow cells. Cultures were performed in triplicate.

Adjuvant activity. F₁-hybrid mice (CBA $\times$ C57BL, 18–20 g) were immunized intravenously with 5 $\times$ 10⁹ sheep red blood cells (SRBC) simultaneously with an intraperitoneal injection of 0.1 or 1.0 μ g of freeze-dried *B. subtilis* cells, LTA, PG-TA or LPS (*E. coli* O26:B6, Sigma). Control mice received SRBC only. Spleen cells were collected on day 4 in cold Hanks' BSS, filtered and adjusted to 10⁷ viable cells ml⁻¹. Antibody-forming cells were counted according to Jerne & Nordin (1963) as plaque-forming cells. SRBC were added to a 0.7% agarose (Sigma) solution and mixed with 10⁶ spleen cells of each mouse. The mixtures were poured into dishes, incubated at 35 °C in the presence of fresh guinea-pig complement, and plaque-forming cells were enumerated.

Statistical methods. Student's *t*-test was used for the calculation of significance; *P* < 0.05 was considered significant.

Results and Discussion

Chemical composition

Chemical analyses of the isolated LTA revealed a composition typical for *Bacillus* (Iwasaki *et al.*, 1986, 1989). The main components were glycerol, phosphate,

Table 1. Chemical composition of the purified PG-TA and LTA of *Bacillus subtilis* IH6564

Component	Composition ($\mu\text{mol mg}^{-1}$)	
	PG-TA	LTA
Glucose	1.6	0.13
Ribose	< 0.003	< 0.003
Fatty acids*	0.0041	0.16
Phosphate	1.7	1.9
Hexosamines	0.89	0.73
DAP	0.39	< 0.001
Alanine	0.53	< 0.002
Glycerol	1.4	1.8
Sodium	1.3	1.4
Lowry-positive material ($\mu\text{g mg}^{-1}$)	70	17

* Calculated using the molecular mass of pentadecanoic acid (242 Da).

Table 2. *In vitro* influence on the number of murine bone marrow granulocyte-monocyte colony-forming cells of freeze-dried *B. subtilis* cells, LTA and PG-TA

The preparations were mixed with normal BALB/c mouse bone marrow cells, the suspensions added to 0.9% agar and dispensed into culture wells to which a standard GM-CSF-containing material had been added. The cultures were incubated for 7 d at 37 °C, and the number of colonies was counted.

Dose ($\mu\text{g ml}^{-1}$)	Colony-forming cells (mean $\pm$ SEM)		
	<i>B. subtilis</i> cells	LTA	PG-TA
0.1	29 $\pm$ 2.4	28 $\pm$ 3.3	28 $\pm$ 4.1
1.0	30 $\pm$ 2.9	45 $\pm$ 4.8	30 $\pm$ 3.9
10	19 $\pm$ 3.2	15 $\pm$ 1.6	29 $\pm$ 2.3
100	20 $\pm$ 3.9	0	34 $\pm$ 4.1
Controls:	medium with GM-CSF	26 $\pm$ 1.8	
	medium without GM-CSF	0	

fatty acids and glucose (Table 1). There was also a considerable amount of hexosamine, but the amount of alanine was below detection limit. No PG-TA was present in the LTA preparation, since the amount of DAP was below the detection limit.

The chemical composition of the isolated PG-TA (Table 1) also corresponded to the basic structure of its PG-glycero-TA moieties. The molar ratio of hexosamines to DAP was about 2:1, and that of glycerol to phosphate about 1:1. The amounts of phosphate, hexosamine, DAP, alanine and glycerol (1.7, 0.9, 0.4, 0.5 and 1.4 $\mu\text{mol mg}^{-1}$, respectively) were in agreement with those reported for *B. subtilis* 168 in other laboratories (1.1–1.7, 1.0, 0.4–0.6, 0.5 and 0.7–1.3 $\mu\text{mol mg}^{-1}$, re-

spectively; Fein & Rogers, 1976; Pooley *et al.*, 1987; Briehl *et al.*, 1989). The purified PG-TA contained traces of membrane-derived material as demonstrated by the detection of 0.004 μmol fatty acids mg^{-1} .

No ribose was detected in either LTA or PG-TA, thus excluding the presence of RNA. Semi-quantitative assay on ethidium bromide plates was negative, indicating that the amount of DNA in both LTA and PG-TA was less than 1 $\mu\text{g mg}^{-1}$. The approximately 1:1 molar ratio of phosphate to sodium in both LTA and PG-TA showed that they were present in the sodium salt form.

Acute toxicity

The toxicity of *B. subtilis* cells, LTA and PG-TA was tested by injecting up to 200 μg of the preparations in mice and up to 1000 μg in guinea-pigs. None of the preparations caused weight loss or symptoms of illness in the animals during 7 d. It was thus concluded that the preparations were devoid of acute toxicity.

Pyrogenicity and *Limulus* activity

B. subtilis cells, LTA and PG-TA were pyrogenic in rabbits at doses of 120, 30 and 30 $\mu\text{g kg}^{-1}$, respectively and non-pyrogenic at doses of 12, 3 and 3 $\mu\text{g kg}^{-1}$, respectively. The pyrogenicity of PG-TA is in accordance with previous reports on other peptidoglycans (Heymer & Rietschel, 1977; Stewart-Tull, 1980). Since LTA, on the other hand, has been described as non-pyrogenic (Wicken & Knox, 1980), we used the LAL-assay to test the possibility that our preparations contained LPS as a contaminant responsible for the observed pyrogenicity. The minimal concentrations of LTA, PG-TA and standard LPS that yielded a positive LAL-reaction were 0.3, 30 and 6.8×10^{-6} $\mu\text{g ml}^{-1}$, respectively. Assuming that all LAL-positive material was LPS, we calculated the maximal amount of LPS present in the preparations, and from it the maximal amount of LPS per pyrogenic dose. This turned out to be 0.7 ng for LTA and 0.007 ng for PG-TA. By comparing these data with the minimal pyrogenic dose of LPS (of the order of 1 ng kg^{-1} ; Kuronen *et al.*, 1977; Muotiala *et al.*, 1992), we concluded that the temperature rise seen in rabbits injected with LTA could have been due to contaminating LPS, whereas that seen with PG-TA was clearly due to its inherent pyrogenicity. Since a positive reaction in the LAL-assay has been claimed to be not LPS-specific but rather a result of the unique reactivity of amphipathic molecules (Wicken & Knox, 1980), we cannot exclude the possibility that the temperature rise seen by using LTA could also have been due to its inherent pyrogenicity.

Fig. 1. *In vivo* influence on the number of murine bone marrow granulocyte-monocyte colony-forming cells of *B. subtilis* cells (a), purified LTA (b) and PG-TA (c). Mice were injected intraperitoneally with the preparations [(○) 0.1 µg per mouse; (●) 1.0 µg per mouse] and killed at different times. Cell suspensions of their bone marrow cells were prepared, mixed with 0.9% agar and dispensed into plastic dishes to which a standard GM-CSF-containing material had been added. The cultures were incubated for 7 d at 37 °C, and the number of colonies was counted. The horizontal bar represents the control level obtained without injecting any of the preparations (mean of all the points $\pm$ SEM).

Mitogenicity

We quantified the mitogenicity of our preparations as their ability to stimulate the incorporation of [3 H]thymidine in mouse spleen cells. LPS and ConA were used as standard mitogens at their optimal concentrations (100 µg ml $^{-1}$ and 2 µg ml $^{-1}$, respectively), and were found to cause a 15–20-fold stimulation compared to the culture incubated with medium only. At the highest test concentration of 100 µg ml $^{-1}$, *B. subtilis* cells showed a weak – about twofold – and PG-TA about fourfold stimulation. We found no mitogenicity in LTA at any concentration (0.01–100 µg ml $^{-1}$); instead, concentrations of 1–100 µg ml $^{-1}$ showed a suppressing, probably cytotoxic effect (to be discussed below). According to Beachey *et al.* (1979), streptococcal LTA is a mitogen for human and mouse lymphocytes, especially T cells. The effect on mouse cells was reported to be strongly concentration-dependent, with a stimulation at 0.005–0.05 µg ml $^{-1}$, and suppression at 0.5–50 µg ml $^{-1}$.

GM-CSF responses

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is defined as a molecule able to stimulate the proliferation of bone marrow progenitors of granulocytes and macrophages to form colonies on *in vitro* culture (for a review, see Metcalf, 1990). GM-CSF stimulates the generation of erythroid and myeloid cells in the bone marrow and regulates their functions. Well-known substances capable of inducing the production of GM-CSF are LPS (Metcalf, 1971; Quesenberry *et al.*,

1972; Moore *et al.*, 1979), phytohaemagglutinin and monokines such as IL-1 and TNF (Gasson, 1991). LPS also directly affects colony formation by increasing the responsiveness of progenitor cells to GM-CSF (Moore *et al.*, 1980). There are few data on the effects of LTA or PG on the induction of GM-CSF, but PG from *Escherichia coli* (Staber & Metcalf, 1980) and synthetic muramyl dipeptide (Galelli & Chedid, 1983) have been shown to increase production of GM-CSF *in vivo*.

In vitro studies. We first investigated the effect of *B. subtilis*-derived components on the *in vitro* responsiveness of colony-forming cells to GM-CSF. Upon addition to a culture of mouse bone marrow cells incubated with a standard dose of GM-CSF, LTA at a concentration of 1.0 µg ml $^{-1}$ induced an increase in the number of colonies, whereas neither *B. subtilis* cells nor PG-TA had such an effect (Table 2). The effect of LTA was, however, weak in comparison to LPS, which has been reported to possess a maximal stimulating effect at concentrations of 0.1–10 ng ml $^{-1}$ (Moore *et al.*, 1979, 1980). As a further control, mouse bone marrow cells were incubated under the same conditions but without GM-CSF; none of the preparations induced any colony-forming cells (data not shown).

At higher concentrations, LTA brought about a dramatic drop in the number of colonies (Table 2), suggesting a cytotoxic effect. Other authors have also described cytotoxicity of high concentrations (50 µg ml $^{-1}$ or more) of LTA in cultures of murine splenocytes (Bolton, 1981), mouse fibroblasts (Leon & Panos, 1983), and human heart cells (Courtney *et al.*, 1986). Courtney *et al.* (1986) and Sutcliffe & Shaw (1991) have suggested

Table 3. Adjuvant effect of freeze-dried *B. subtilis* cells, LTA and PG-TA on anti-SRBC plaque-forming cell generation of F_1 -hybrid (CBA $\times$ C57BL) mice *in vivo*

Three mice in a group were injected simultaneously with SRBC and the appropriate preparation. On day 4 the spleens were collected in buffer, pooled, mixed with SRBC, and incubated on dishes at 35 °C. Plaques were counted to give the number of antibody-forming cells (PFC-number).

Stimulation index = mean PFC numbers in three replicate spleen cell cultures of the experimental group/mean PFC numbers in three replicate cultures of the control group. Number of mice tested is shown in parentheses.

Component	Dose (μ g)	Stimulation index			
		Expt 1	Expt 2	Expt 3	Mean values
<i>B. subtilis</i> cells	0.1	1.12 (5)	0.75 (5)	ND	0.94 (10)
	1.0	1.26* (5)	1.39* (5)	ND	1.33* (10)
LTA	0.1	1.39* (5)	ND	1.55* (5)	1.47* (10)
	1.0	1.00 (6)	ND	1.11 (4)	1.06 (10)
PG-TA	0.1	1.25* (6)	0.83 (3)	0.97 (5)	1.02 (14)
	1.0	1.31* (5)	1.21* (4)	1.20 (4)	1.24* (13)
<i>E. coli</i> (O26:B6) LPS	0.1	1.13 (5)	1.88* (5)	1.56* (3)	1.48* (13)
	1.0	2.81* (5)	3.31* (5)	1.84* (4)	2.65* (14)

* Indicates a significant difference between the control and experimental groups ($P < 0.05$).
ND, Not determined.

that LTA may be cytotoxic for cells only above its critical micellar concentration. This could explain the inhibition of colony formation seen at high concentrations of our LTA-preparation, since the critical micellar concentration of LTA from several species of bacteria has been reported to be relatively high (28–60 μ g ml⁻¹ in phosphate-buffered saline; Courtney *et al.*, 1986).

In vivo studies. *B. subtilis* cells, LTA and PG-TA were injected into BALB/c mice at a concentration of 0.1 μ g or 1.0 μ g, and the number of colony-forming cells in their bone marrow was determined after 16 h and 1, 2, 5 and 7 d (Fig. 1). Each of the preparations induced an increase in the numbers of bone marrow colony-forming cells, best seen between days 2 and 5. In most experiments, the effect was already disappearing on day 7. The increase in colony numbers was probably due to an increase in the production of GM-CSF.

Adjuvant activity

We found evidence for adjuvant activity with each of the preparations (Table 3). Both *B. subtilis* cells and PG-TA had a small but statistically significant adjuvant effect at the larger dose (1.0 μ g per mouse). LTA increased the SRBC-specific plaque-forming cell response only at the lower dose (0.1 μ g per mouse). Other adjuvants, including lipid-free LTA, have also been shown to enhance or suppress the immune response depending upon the dose and timing (Chorpenning *et al.*, 1979). LPS stimulated the plaque-forming cell response at both concentrations (0.1 and 1.0 μ g per mouse) tested, in a

dose-dependent manner. A decrease in response was not observed with any dose of any of the substances tested.

Concluding remarks

In this study we assessed the biological activities of purified and chemically characterized LTA and PG-TA from a derivative of *B. subtilis* 168 (Marburg), which is a suitable host for producing heterologous vaccine proteins and other pharmaceuticals. Previous reports of biological activities of LTA and PG-TA contain many discrepancies, probably due to different sources and hence different chemical structures of the preparations used. In particular, the 2-OH substitution and the salt form of glycerol-phosphate polymers may influence the responses seen in the biological assays. Thus, a proper chemical characterization of the preparations used was essential, especially since sensitive and accurate analytical methods are available nowadays.

LTA and PG-TA were found to be non-toxic. PG-TA and possibly LTA were weakly pyrogenic. Putative heterologous vaccine proteins obtained from derivatives of *B. subtilis* 168 (Marburg) have been estimated to contain 1% or less of PG-TA and 4% or less of LTA (Himananen *et al.*, 1993). One human vaccine dose (estimated as 50 μ g protein) would thus contain 0.5 μ g or less of PG-TA and 2 μ g or less of LTA. On the basis of our present results, these amounts are clearly below the pyrogenic dose of PG-TA and LTA (30 μ g). These amounts of both LTA and PG-TA could have immunostimulatory effects: in mice a dose of 0.1 and/or 1.0 μ g of

both compounds increased the number of bone marrow granulocyte-monocyte colony-forming cells and exhibited adjuvant activity for antibody production. The level and duration of the stimulatory effect of bacillar components upon GM-CSF production is of interest, because of its probable role in inducing resistance to viruses (human immunodeficiency virus; Groopman *et al.*, 1987) and bacteria (*Salmonella typhimurium*; Morrissey *et al.*, 1989). Hence, in respect to vaccine production the minute amounts of LTA and PG-TA in the purified heterologous proteins produced in *B. subtilis* 168 (Marburg) are not expected to cause harmful effects; instead, they may have beneficial effects on host defence.

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