

## Induction of Nitric Oxide Production by Polyosides from the Cell Walls of *Streptococcus mutans* OMZ 175, a Gram-Positive Bacterium, in the Rat Aorta

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Received 7 October 1996/Returned for modification 3 January 1997/Accepted 5 March 1997

**The cardiovascular dysfunctions associated with septic shock induced by gram-negative or gram-positive bacteria (gram-positive or gram-negative septic shock) are comparable. In gram-negative septic shock, lipopolysaccharide (LPS) induces nitric oxide (NO) synthase, which contributes to the vascular hypotension and hyporeactivity to vasoconstrictors. The role of NO in gram-positive septic shock and the nature of the bacterial wall components responsible for the vascular effects of gram-positive bacteria are not well known. This study investigated the vascular effects of cell wall serotype polyosides, rhamnose glucose polymers (RGPs), from *Streptococcus mutans*, in comparison with lipoteichoic acid (LTA) from *Staphylococcus aureus*, on the induction of NO synthase activity in the rat aorta. We show that 10 µg of both RGPs and LTA per ml induced hyporeactivity to noradrenaline, L-arginine-induced relaxation, increases of 2.2- and 7.8-fold, respectively, of cyclic GMP production, and increases of 7- and 12-fold in nitrite release. All of these effects appeared after several hours of incubation and were inhibited by *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase. Electron paramagnetic resonance spin trapping experiments demonstrated directly that RGPs and LTA induced NO overproduction (four- to eightfold, respectively) in rat aortic rings; this production was inhibited by L-NAME and prevented by dexamethasone. These results demonstrate directly the induction of NO production in vascular tissue by LTA and show that another, chemically different component of gram-positive bacteria can also have these properties. This result suggests that different components of the gram-positive bacterial wall could be implicated in the genesis of cardiovascular dysfunctions observed in gram-positive septic shock.**

Septic shock is an urgent medical problem, with more than 200,000 cases occurring each year with an associated mortality rate of 20 to 80% in the United States (39). Septic shock can be induced by gram-negative as well as by gram-positive bacteria. Up to now, the importance of gram-negative organisms in the genesis of sepsis has been emphasized. However, recent studies show an increasing incidence of gram-positive sources of sepsis (6).

Lipopolysaccharide (LPS; the glycolipid part of endotoxin), a component of the bacterial cell wall (41), is considered the single contributor involved in eliciting gram-negative sepsis. Administration of endotoxin in animals (37) or humans (51) reproduces the signs and symptoms of sepsis. LPS by itself and/or through releasing a number of cytokines causes expression of inducible nitric oxide synthase (i-NOS) in a wide variety of cells and tissues. Overproduction of nitric oxide (NO) as a result of i-NOS activity in vascular tissue contributes greatly to hypotension and hyporeactivity associated with endotoxin shock (49, 52).

The cardiovascular dysfunctions, the inflammatory responses, and the level of mortality associated with gram-positive or gram-negative bacterium-induced sepsis (gram-positive or gram-negative sepsis) seem to be comparable in spite of the absence of LPS in the gram-positive bacterial cell wall (1, 7, 24). Enhanced formation of NO may also contribute to the

circulatory failure in gram-positive sepsis. This is supported by findings demonstrating that (i) killed *Staphylococcus aureus* (10) can induce the expression of i-NOS in macrophages and (ii) lipoteichoic acid (LTA), a component associated with the membrane of gram-positive bacteria (60), can induce the expression of i-NOS in cultured vascular smooth muscle cells (31) or in macrophages (30) in vitro. In addition, the delayed circulatory failure caused by LTA in the rat was prevented by continuous infusion of *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME; an inhibitor of NO synthase) (12). LTA has therefore been implicated as the major component of the gram-positive bacterial cell wall which is itself and/or via cytokine release responsible for the induction of i-NOS.

Some data suggest that in addition to LTA, some other component(s) of the gram-positive bacterial wall may contribute directly or indirectly to the induction of i-NOS. Another bacterial wall component, peptidoglycan G, does not induce i-NOS but enhances greatly the effect of LTA (13). Bacterial serogroup polyosides of *Streptococcus mutans* OMZ 175 (rhamnose glucose polymers [RGPs]) induce the release of several cytokines, including tumor necrosis factor alpha (TNF-α) (3), which is known to be a powerful inducer of i-NOS activity (18, 36, 37, 45).

While indirect data suggest that i-NOS activity can be induced by LTA in vascular tissue, actual elevated NO levels in this situation have not been measured directly. In this study, we applied the electron paramagnetic resonance (EPR) spin trapping approach to characterize semiquantitatively NO production (34, 56) in order to investigate NO level in LTA-treated vascular tissue. The long-term influence of RGPs on the contractile property of vessels is not known. Using both indirect

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and direct approaches, we studied the effects of RGPs, another component of the wall of gram-positive bacteria, on the induction of i-NOS activity in rat aorta and compared their effects with those of LTA and of LPS.

#### MATERIALS AND METHODS

**Materials.** Drugs were obtained from Sigma, St. Louis, Mo. All cell culture media used were obtained from Gibco BRL Life Technology, Cergy Pontoise, France. The LPS (*Escherichia coli* O55:B5) used was from Difco, Detroit, Mich. LTA from *S. aureus* was obtained from Sigma.

RGPs were prepared from lyophilized *Streptococcus mutans* OMZ 175 (serotype f) by the method of Hamada et al. (20), modified by Benabdelmoumène et al. (3). Briefly, polysaccharides were obtained from supernatant of autoclaved (30 min, 120°C) bacterial cells, and charged components were eliminated by chromatography on a DEAE-Trisacryl M column equilibrated with a 0.01 M Tris-HCl buffer (pH 8.2). Unadsorbed fractions were assayed for proteins, carbohydrate, and phosphorus, and samples containing only carbohydrates were incubated with CNBr-panose (24 h at 25°C) and further chromatographed on a DEAE-Trisacryl column as described above (3). The serotype polysaccharides used in this study had a molecular weight of  $6 \times 10^5$ , contained >98% neutral sugars by weight, were free of endotoxin (less than 0.00003%) by *Limulus* and pyrogenicity tests, and had only trace amounts of protein (<0.05%) and phosphorus (<0.01%).

**Functional studies. (i) Effects of RGPs on the contractile response to NA of rat aortic rings.** Male Wistar rats (11 weeks old) were killed by cervical dislocation. The thoracic aorta was removed and cleaned of adherent tissues, and 2-mm-long rings were prepared. Rings with endothelium were incubated for 4 h with RGPs (10 µg/ml) in Dulbecco's modified Eagle's medium at 37°C in a humidified incubator gassed with 5% CO<sub>2</sub> in air. The endothelium was removed just before the rings were mounted in organ bath chambers as previously described (26). The absence of functional endothelium is checked by the failure of acetylcholine (1 µmol/liter) to relax rings precontracted with noradrenaline (NA; 1 µmol/liter). After a washing period of at least 1 h, during which the tension returned to its basal level, contractility was evaluated by cumulative addition of NA (1 nmol/liter to 3 µmol/liter) 8 h after the first contact with the bacterial products (*t*<sub>8 h</sub>). The pD<sub>2</sub> {−log<sub>10</sub> [50% effective dose (moles/liter)]} and the maximal effect of NA (*E*<sub>max</sub>; grams/milligram of dried tissue) were calculated.

**(ii) Effect of RGPs on the vasomotor response of precontracted aortic rings to L-arginine.** When the maximal tension was stable (stable tension after the addition of 3 µmol of NA per liter), the effect of 1 mmol of L-arginine per liter was tested. The relaxation induced by L-arginine on precontracted rings is an index of the induction of i-NOS (49). When the tension after L-arginine addition was stable, 1 mmol of L-NAME per liter was added. Thereafter, when this latter effect of L-NAME was stabilized, 3 µmol of methylene blue (MeB; an inhibitor of the activation of the soluble guanylyl cyclase) per liter was added. Results are expressed as tension developed (grams) per milligram of dried tissue, and values shown are the means ± standard errors of the means of *n* experiments.

**(iii) Influence of the endothelium on the effects of RGPs on the vasomotor responses to NA and to L-arginine.** Rat aortic rings with or without endothelium were incubated for 4 h with RGPs (10 µg/ml) as described above. Rings with endothelium were gently rubbed with forceps in order to remove the endothelium just before they were mounted in organ bath chambers. The contractile response to NA and the effect of L-arginine on precontracted rings were tested at *t*<sub>8 h</sub>.

**Biochemical effects of RGPs and LTA on rat aortic rings. (i) Determination of tissue cyclic GMP content.** Rat aortic rings with endothelium were incubated for 4 h in MH10 (minimal essential medium-Ham F10 [vol/vol]) with saline, RGPs (10 µg/ml), LTA (10 µg/ml), or LPS (10 µg/ml). The endothelium was then removed, and rings were placed in baths with bubbled Krebs Henseleit (in millimoles per liter: NaCl, 118.4; KCl, 3.7; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; KH<sub>2</sub>PO<sub>4</sub>, 2.2; NaHCO<sub>3</sub>, 24.9; and glucose, 10) with 0.1 mmol of L-arginine per liter and with or without L-NAME (1 mmol/liter) for 4 h. Isobutylmethylxanthine (a nonselective inhibitor of phosphodiesterases; 100 µmol/liter) was added to rings 30 min before *t*<sub>8 h</sub>. At *t*<sub>8 h</sub>, rings were frozen in liquid nitrogen and then placed in 1 ml of 0.1 N HCl. Following homogenization with a glass-glass potter and sonication, the samples were centrifuged. Pellets and supernatants were frozen and kept at −20°C. The cyclic GMP content was determined in the supernatant by using a radioimmunoassay previously described (9), modified by separation of the free cyclic GMP with activated charcoal. Protein content was determined in the pellet according to the Bradford method (8), with bovine serum albumin as the standard.

**(ii) Assay of NO production.** Rat aortic rings with endothelium were incubated for 16 h in Dulbecco's modified Eagle's medium at 37°C in a gassed (95% air–5% CO<sub>2</sub>) humidified incubator with saline, RGPs (10 µg/ml), LTA (10 µg/ml), or LPS (10 µg/ml). The incubation medium was used for nitrite determination, while the rings were used to measure NO production by spin trapping followed by EPR spectroscopy.

**(iii) Nitrite assay.** The accumulation of nitrite was measured in the incubation medium by the Griess reaction. One hundred microliters of Griess reagent [1% sulfanilamide–0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% orthophosphoric acid] was added to 100 µl of medium. After 10 min of incubation

at room temperature, the absorbance of the mixture at 545 nm was measured. Concentrations were determined relative to a standard curve, using sodium nitrite. The amount of nitrite produced was expressed as nanomoles/milligram of dried tissue per 16 h.

**(iv) Spin trapping of NO and EPR spectroscopy.** For direct detection of NO in rat aortic rings, we used a previously described NO spin trapping approach based on the ability of Fe(DETC)<sub>3</sub> to react with NO to form NO-Fe(DETC)<sub>2</sub> adducts, which are characterized by an axial EPR signal with  $g_{\perp} = 2.035$  and  $g_{\parallel} = 2.02$  and triplet hyperfine splitting of 1.3 mT at  $g_{\perp}$  (35, 56). Briefly, after 16 h of incubation, rings were placed in 1 ml of fresh medium and incubated with sodium diethyldithiocarbamate (DETC; 5 mmol/liter), FeSO<sub>4</sub> · 7H<sub>2</sub>O (50 µmol/liter), and sodium citrate (1 mmol/liter) at 37°C for 1 h. Rings were then frozen and kept in liquid nitrogen. EPR spectra were recorded on a Bruker 300E spectrometer at 77 K, using a Dewar flask (Wilmad). EPR settings were 10 mW of microwave power, 0.61 mT of amplitude modulation, a 9.45-GHz microwave frequency, and a 100-kHz modulation frequency. For quantification of NO-Fe(DETC)<sub>2</sub> formed in tissue, a solution of paramagnetic (NO)<sub>2</sub>Fe(S<sub>2</sub>O<sub>8</sub><sup>2-</sup>) of known concentration was used (35, 56). The contents of NO-Fe(DETC)<sub>2</sub> formed were normalized per milligram of dried aortic tissue.

**Statistics.** Results are expressed as means ± standard errors of the means of *n* experiments. Statistical significance was tested by one-way or multiple analysis of variance followed by a posteriori Kruskal-Wallis and Mann-Whitney tests when a significant difference was detected.

#### RESULTS

**Effects of RGPs and LTA on the contractile response to NA and L-arginine.** In vitro, both LPS (26) and LTA (31) are able to decrease the contractile response of the rat aorta to NA. We have investigated the effect of RGPs in a similar experimental setup. In preliminary experiments, RGPs induced a reproducible vascular hyporeactivity at a concentration of 10 µg/ml. This concentration was previously shown to induce the secretion of cytokines from monocytes (48) and was therefore used throughout this study. After incubation of rat aortic rings for 4 h in vitro with 10 µg of RGPs per ml, the concentration-response curve for NA was shifted to the right (Fig. 1a). The pD<sub>2</sub> for NA after RGP treatment was significantly decreased ( $8.6 \pm 0.1$  and  $7.9 \pm 0.1$  for control and treated rings, respectively;  $P < 0.01$ ;  $n = 7$  for each group). However, the *E*<sub>max</sub> obtained with 3 µmol of NA per liter was not modified by RGPs ( $3.6 \pm 1.2$  and  $3.5 \pm 0.1$  g · mg of dried tissue<sup>−1</sup> for control and treated rings, respectively).

L-Arginine (1 mmol/liter) induced a significant relaxation on rings treated by RGPs ( $6.3\% \pm 2.3\%$  versus  $24.9\% \pm 3.4\%$  for control and treated rings, respectively;  $P < 0.01$  [Fig. 1b]). This L-arginine-induced relaxation was completely reversed by L-NAME (1 mmol/liter), an inhibitor of NO synthase, or by MeB (3 µmol/liter), an inhibitor of the activation of the soluble guanylyl cyclase by NO.

LTA induced a significant decrease of the pD<sub>2</sub> for NA ( $8.6 \pm 0.1$  and  $7.1 \pm 0.2$  for control and LTA-incubated rings, respectively;  $P < 0.01$ ;  $n = 5$  to 7 [data not shown]) and of *E*<sub>max</sub> ( $3.6 \pm 0.2$  versus  $2.9 \pm 0.2$  g · mg of dried tissue<sup>−1</sup> for control and LTA-incubated rings, respectively;  $P < 0.05$  [data not shown]). The addition of L-arginine induced approximately 56% relaxation on LTA-treated rings.

**Influence of the endothelium on the effects of RGPs.** The presence of the endothelium during the incubation time increased significantly the effects of RGPs on the contractile response to NA. The pD<sub>2</sub> for NA of RGP-treated rings with endothelium was significantly lowered ( $8.5 \pm 0.1$  and  $7.8 \pm 0.1$  for control and RGP-treated rings, respectively;  $P < 0.01$  [results not shown]). When rings were incubated without endothelium, RGPs caused only a small, nonsignificant decrease of the pD<sub>2</sub> for NA ( $8.4 \pm 0.1$  and  $8.2 \pm 0.1$  g · mg of dried tissue<sup>−1</sup> for control and RGP-treated rings, respectively). There was no effect of RGPs on the *E*<sub>max</sub> for NA, whether the endothelium was present or not.

L-Arginine (1 mmol/liter) induced a significant relaxation of RGP-treated rings with endothelium ( $2.8\% \pm 0.8\%$  and  $26.3\%$

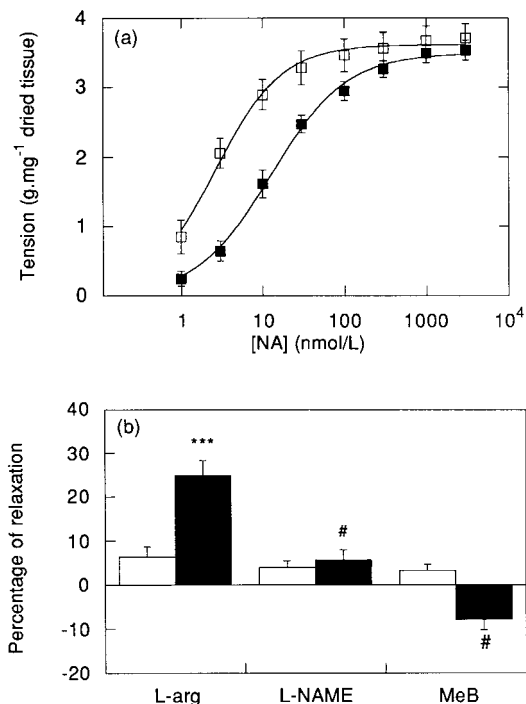


FIG. 1. Effects of RGP on the vasomotor responses to NA (a) and to L-arginine (b). Rings were incubated for 4 h with RGP (10  $\mu\text{g/ml}$ ) (■) or with saline (□). The contractile response to cumulative addition of NA (1 nmol/liter to 3  $\mu\text{mol/liter}$ ) was then tested at  $t_{8\text{h}}$  (a). When the maximal tension (stable tension after the addition of 3  $\mu\text{mol}$  of NA per liter) was obtained, L-arginine (1 mmol/liter) was added, and the percentage of relaxation was recorded (b) (open bars, controls; closed bars, RGP). After the observation of a stabilized relaxant effect of L-arginine, 1 mmol of L-NAME per liter was added; when this latter effect of L-NAME was stabilized, 3  $\mu\text{mol}$  of MeB per liter was added. \*\*,  $P < 0.01$  compared with control; #,  $P < 0.05$  compared with the same treatment before the addition of L-NAME ( $n = 7$ ).

$\pm 3.9\%$  for control and RGP-treated rings, respectively;  $P < 0.01$  [results not shown]). When rings were incubated without endothelium, the L-arginine-dependent relaxation was not significant ( $4.1\% \pm 2.1\%$  and  $7.2 \pm 1.9\%$  for control and RGP-treated rings, respectively;  $n = 6$  to 8).

#### Effect of RGP and LTA on NO generation in aortic rings.

(i) **Effect on cyclic GMP tissue content.** Rat aortic rings with endothelium were incubated with RGP (10  $\mu\text{g/ml}$ ) for 4 h. Intracellular content of cyclic GMP was assayed 8 h after the first contact with the bacterial product. RGP induced a significant 2.2-fold increase of cyclic GMP level ( $12.6 \pm 3.6$  and  $28.3 \pm 4.7$  pmol  $\cdot$  mg of protein tissue<sup>-1</sup> for control and treated rings, respectively;  $n = 9$ ;  $P < 0.05$ ) (Fig. 2). This increase was totally inhibited by an inhibitor of NO synthase, L-NAME.

In parallel, LTA (10  $\mu\text{g/ml}$ ) and LPS (10  $\mu\text{g/ml}$ ) induced, respectively 7.8- and 7.5-fold increases of cyclic GMP production ( $P < 0.01$  compared to the control for each treatment) which were totally inhibited by L-NAME (Fig. 2).

(ii) **Effect on nitrite production.** Incubation of rat aortic rings with endothelium for 16 h with RGP (10  $\mu\text{g/ml}$ ) induced a sevenfold increase in nitrite production ( $P < 0.01$ ) which was prevented by dexamethasone (10  $\mu\text{mol/liter}$ ) (Fig. 3).

LTA or LPS also caused an increase in nitrite formation (12- and 14-fold, respectively;  $P < 0.01$ ) which was also prevented by dexamethasone (Fig. 3).

(iii) **EPR spin trapping experiments: measurement of NO production.** Aortic rings with endothelium incubated for 16 h

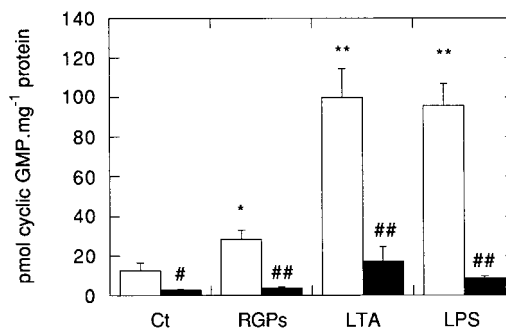


FIG. 2. Effects of bacterial products on the cyclic GMP levels in rat aortic rings. Rat aortic rings were incubated for 4 h with saline (Control [Ct]), RGP (10  $\mu\text{g/ml}$ ), LTA (10  $\mu\text{g/ml}$ ), or LPS (10  $\mu\text{g/ml}$ ) alone (open bars) or with L-NAME (1 mmol/liter; closed bars). The cyclic GMP content of tissue was measured at  $t_{8\text{h}}$  after the first contact with bacterial components. \*,  $P < 0.05$ , and \*\*,  $P < 0.01$  compared with controls; #,  $P < 0.05$ , and ##,  $P < 0.01$  compared with the same treatment but without L-NAME ( $n = 9$ ).

with RGP (10  $\mu\text{g/ml}$ ) and then exposed for 1 h to DETC (5 mmol/liter) and FeSO<sub>4</sub> (50  $\mu\text{mol/liter}$ ) exhibited an axial EPR signal with  $g_{\perp} = 2.035$  and  $g_{\parallel} = 2.02$  and triplet structure at  $g_{\perp}$  (Fig. 4). This signal is characteristic of NO-Fe(DETC)<sub>2</sub> complexes, and its amplitude reflects the amount of NO formed (25, 34, 35, 56). Quantitative analysis showed that RGP induced approximately a fourfold increase in NO-Fe(DETC)<sub>2</sub> complex formation (Fig. 5;  $P < 0.01$ ) which was prevented by dexamethasone (Fig. 5a;  $P < 0.01$ ) and L-NAME (Fig. 5b;  $P < 0.001$ ).

LTA (10  $\mu\text{g/ml}$ )- and LPS (10  $\mu\text{g/ml}$ )-treated rings exhibited the EPR signal of the NO-Fe(DETC)<sub>2</sub> complex (Fig. 4). They induced a four- to eightfold increase of NO-Fe(DETC)<sub>2</sub> content, which was prevented by dexamethasone and L-NAME (Fig. 5).

## DISCUSSION

NO overproduction has been implicated in the vascular hypotension and hyporeactivity to vasoconstrictors observed in septic shock induced by gram-negative bacteria (49). In an *in vivo* model, this induction of NO production in the vascular wall causes a decrease both of the mean arterial blood pressure and of the pressor response to vasoconstrictors such as NA (40, 52, 53); *in vitro*, NO synthase induction causes a decrease of

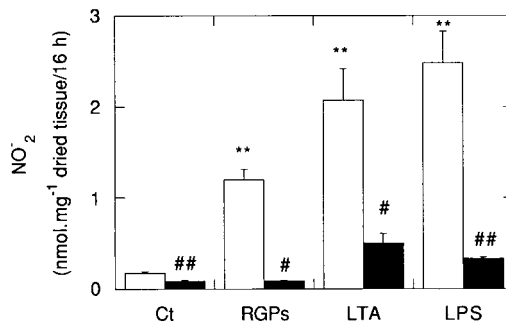


FIG. 3. Effects of bacterial products on nitrite accumulation by rat aortic rings. Rings were incubated for 16 h with saline (control [Ct]), RGP (10  $\mu\text{g/ml}$ ), LTA (10  $\mu\text{g/ml}$ ), or LPS (10  $\mu\text{g/ml}$ ) alone (open bars) or with dexamethasone (10  $\mu\text{mol/liter}$ ; closed bars). The concentration of nitrite in the incubation medium was measured by the Griess reaction. \*\*,  $P < 0.01$  compared with control; #,  $P < 0.05$ , and ##,  $P < 0.01$  compared the same treatment but without dexamethasone ( $n = 10$ ).

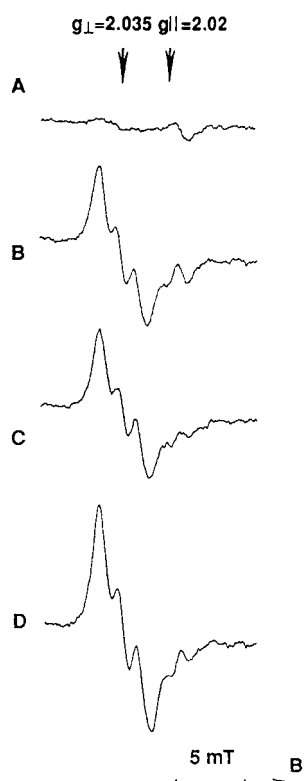


FIG. 4. Representative EPR spectra of rat aortic rings incubated with saline (A), RGPs (10  $\mu\text{g/ml}$ ) (B), LTA (10  $\mu\text{g/ml}$ ) (C), or LPS (10  $\mu\text{g/ml}$ ) (D) for 16 h. After exchange of medium, rings were treated with DETC (5 mmol/liter) and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (50  $\mu\text{mol/liter}$ ) for 1 h. Parameters were as described in Materials and Methods.

the contractile effect of NA (17, 26). All of these effects are inhibited by an inhibitor of NO synthase or by dexamethasone, which prevents the induction of i-NOS in the vascular wall (42).

In gram-positive septic shock, the type of bacterial wall components responsible for the vascular disorders and their cellular effects are not well established. It has been shown that different types of killed gram-positive bacteria or LTAs are able to induce NO synthase activity in vascular smooth muscle cells (11), in macrophages (10, 11), or in an in vivo situation (29, 43, 57, 59). Some capsular polysides from the bacterial wall of *Streptococcus mutans* OMZ 175, RGPs, activate human monocytes to release cytokines (48). Their vascular effects were not known. We therefore decided to explore the effects of RGPs on the NO synthase activity in the vascular wall. First, indirect approaches were used: functional studies, measurement of a stable end product (nitrite), and measurement of mediators generated by NO (cyclic GMP). We have shown that both RGPs and LTA induced in blood vessels hyporeactivity to NA, L-arginine-induced relaxation, increased cyclic GMP production, and nitrite release. All of these effects were inhibited by L-NAME, an inhibitor of NO synthase; the production of nitrite was also prevented by dexamethasone, an inhibitor of the induction of i-NOS (42). These data suggest strongly that RGPs induce NO synthase activity in the vessel wall as well as LTA. Some of the effects of LTA described here have been observed in different experimental systems. Lonchampt et al. have shown an increase in cyclic GMP production by smooth muscle cells and a decrease of the contractile response to NA of rat aortic rings (2, 31); LTA induces nitrite production in vascular smooth muscle cells in culture (11), and in vivo, LTA

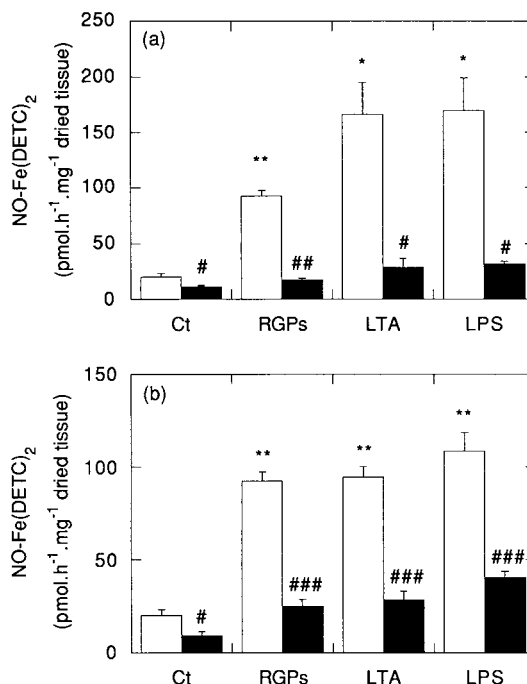


FIG. 5. Effect of bacterial products on NO production estimated by an EPR spin trapping technique. The amount of NO was measured as NO-Fe(DETC)<sub>2</sub> formed during a 1-h incubation of aortic rings with DETC (5 mmol/liter) and  $\text{FeSO}_4$  (50  $\mu\text{mol/liter}$ ). (a) Rings were incubated for 16 h with saline (control [Ct]), RGPs (10  $\mu\text{g/ml}$ ), LTA (10  $\mu\text{g/ml}$ ), or LPS (10  $\mu\text{g/ml}$ ) alone (open bars) or with dexamethasone (10  $\mu\text{mol/liter}$ ; closed bars). \*,  $P < 0.05$ , and \*\*,  $P < 0.01$  compared with the control; #,  $P < 0.05$ , and ##,  $P < 0.01$  compared with the same treatment but without dexamethasone ( $n = 4$  to 8). (b) Similar experimental conditions, but 30 min before the assay, L-NAME (1 mmol/liter) was added (closed bars). \*\*,  $P < 0.01$  compared with the control; #,  $P < 0.05$ , and ###,  $P < 0.001$  compared with the same treatment but without L-NAME ( $n = 7$  to 8).

induces a hypotension and a hyporesponsiveness to NA that seem to be due to the induction of NO synthase activity in the vascular wall (12). For the first time, however, our results provide all of these indirect pieces of evidence in one single homogeneous blood vessel model.

However, these results lacked direct evidence of NO production. Therefore, we used the EPR spin trapping technique to directly detect NO. We show that RGPs, LTA, and LPS induce the delayed increase in NO formation in the vascular wall, thus confirming the indirect evidence described before. The amount of NO produced after contact with RGPs or LTA and the inhibitory effect of L-NAME or dexamethasone were comparable with the effects of LPS observed in the same system.

Up to now, the only component of the gram-positive bacterial cell wall whose action on the induction of NO synthase was well characterized was LTA. We show that a second component of gram-positive bacteria, RGPs, is able to induce NO synthase activity. In contrast, peptidoglycan G, another cell wall component, is not effective as a single component but only increases the effects of LTA (13).

A putative contamination by LPS could explain the present results. However several lines of evidence suggest that it is not the case: (i) the cell wall of *Streptococcus mutans* does not contain endotoxin in its structure; (ii) sterile conditions and apyrogenic reagents were used throughout the purification procedures, so that LPS contamination during this purification process seems unlikely; and (iii) a *Limulus* assay performed on the final RGP preparation was negative, indicating that the

endotoxin level is less than 0.125 endotoxin unit/ml (<0.01 ng/ml). LTA contamination is also ruled out by the low phosphorus content of the RGPs (<0.01%).

The effect of RGPs can be observed only after 8 h, indicating a delayed cellular process and suggesting the induction of protein synthesis. Indeed, the effects of RGPs on nitrite and NO production were abolished by incubation with dexamethasone, which is known to prevent the induction of i-NOS by endotoxin (42) and by LTA (12). The delayed action of RGPs seems to be dependent, at least in part, on the presence of the endothelium. After 8 h, the hyporeactivity to NA and the relaxant effect of L-arginine were significant only when rings were incubated with their endothelium. This suggests that, if not essential for the induction of vascular hyporeactivity, the endothelium was able to sensitize the rat aortic rings to RGPs. Fleming et al. have shown that the presence of endothelium accelerated the induction of i-NOS by LPS and also increased the sensitivity of the preparation to LPS (17). It would be interesting to study the mechanism(s) by which endothelium increases the sensitivity of blood vessels to RGPs. It has recently been shown that capsular polysaccharides of *S. aureus* bind specifically to human monocytes and induce the release of TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and IL-8 (47). RGPs could also induce the release of cytokines by the endothelial cells; those cytokines would accelerate NO synthase induction in the vascular wall, as already shown for TNF- $\alpha$  (18, 36, 45) and IL-1 (27, 28).

What is the importance of this study in understanding the mechanisms of altered vascular reactivity observed in septic shock? LTA has been implicated as the major mediator of gram-positive shock (12). In our study, we observed that the effects of RGPs on NO synthase activity were comparable to those of LTA. It thus seems conceivable that RGPs could be another component of gram-positive bacteria playing an important role in the genesis of the vascular disorders in gram-positive shock. The underlying mechanisms of the vascular effects of RGPs in septic shock might be similar with those induced by LTA and/or LPS; both have been shown to cause directly, but also through cytokine release, the induction of i-NOS in the vessel wall (16, 23, 29, 38, 54, 58). RGPs also cause the release of various cytokines from monocytes (48), thus supporting this hypothesis. In gram-positive septic shock, the cytokines released seem to be comparable to those released during gram-negative septic shock (14, 19, 23, 29, 33, 44, 46, 55). An antibody to TNF and an antagonist of the platelet-activating factor (PAF) receptor have similar protective effects in gram-positive (14, 23) and gram-negative (16, 21, 32, 54) septic shock. However the mechanisms of action of cytokines might be different in the two types of shock. For example, in gram-positive shock, it seems that intracellular PAF is implicated in vascular disorders, while in gram-negative shock, it is the PAF released from cells (14). We observed that the kinetics of induction of NO synthase by RGPs was slower than that of induction by LTA: the contractile response to NA was significantly decreased 6 h after the first contact with LTA but not with RGPs (data not shown). However, this difference disappeared with longer times of incubation. Also, RGPs induced a lower increase in cyclic GMP accumulation than LTA (Fig. 2). This result suggests that the mechanisms activated by RGPs could differ from those of LTA at one or several stages. It has been reported that the induction of NO synthase by some gram-positive bacteria can be long lasting (4, 5, 50), whereas it can be a short-lasting process with some others (10, 61), suggesting that various mechanisms are implicated.

The identification of the compounds implicated in the pathological activity of the gram-positive bacteria will allow us

to explore more precisely the cellular membrane receptors and the intracellular mechanisms activated. The cellular effects of gram-positive bacterial products may (15) or may not (47) be dependent on the presence of serum. Heumann et al. observed that the stimulating activity of the peptidoglycan and the glycan-teichoic acid complex on monocytes required a serum- and/or plasma-derived factor(s) (22). Results described here were obtained in the absence of serum. It has already been determined that on monocytes, the CD14, the C<sub>3</sub>, and the C<sub>1q</sub> receptors bind RGPs: the CD14 receptor mediates cytokine release; the C<sub>3</sub> receptor may mediate the activation of the complement system as well as the clearance of the polyosides as does the C<sub>1q</sub> receptor (48). The membrane receptors involved in the vascular effects of RGPs remain to be investigated.

Our data provide indirect and direct evidence, through functional studies and the EPR spin trapping technique, that both RGPs, polyosides from the bacterial wall of *Streptococcus mutans*, and LTA induce NO synthase activity in the vascular wall. This work supports the hypothesis that different wall components of gram-positive bacteria are involved in the pathogenesis of the vascular disorders observed in septic shock. In this respect, it would be interesting to investigate the complementary vascular effects of various gram-positive bacterial cell wall components.

#### ACKNOWLEDGMENTS

This work was supported in part by grants from the Fondation de France and from the EEC (contract BMH1-CT92-1893.EEC). V. Martin benefitted from a fellowship from the Fondation pour la Recherche Médicale.

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