Inability of the *Francisella tularensis* Lipopolysaccharide To Mimic or To Antagonize the Induction of Cell Activation by Endotoxins

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We studied the ability of the lipopolysaccharide (LPS) extracted from a vaccine strain of *Francisella tularensis* (LPS-Ft) to mimic LPSs from other gram-negative bacteria for activation of various murine cell types or to antagonize the effects of other LPSs. We found that activation of macrophages for the production of tumor necrosis factor alpha and NO, of pre-B lymphocytes for the expression of surface immunoglobulins, and of bone marrow cells for the expression of LPS-binding sites was either undetectable with LPS-Ft or required concentrations 100 to 1,000 times higher than for standard LPSs. Preexposure of macrophages to LPS-Ft also failed to trigger down-regulation of tumor necrosis factor alpha (desensitization) or up-regulation of NO responses to an endotoxin challenge. In contrast to other atypical LPSs, LPS-Ft was also unable to antagonize any of the endotoxin-induced cellular responses mentioned above, suggesting that this LPS does not interact with LPS receptors.

Tularemia is a zoonotic disease caused by the facultative intracellular bacterium *Francisella tularensis*. The species can be divided in two main subspecies, type A and type B. Type A is more virulent than type B. A vaccine strain has been isolated from a type B strain and used for restricted vaccination of humans. The vaccine strain is designated *F. tularensis* LVS (live vaccine strain) (30). *F. tularensis* is highly virulent for humans since only a few microorganisms can cause disease (1, 34). Tularemia, which affects humans and many other mammals, is often conveyed by ticks, logomorphs, or rodents (21, 34). The disease is endemic only in northern and temperate regions of the Northern Hemisphere. The fatality rate of this disease is rather high (5 to 7%), with different clinical manifestations that include cutaneous, glandular, ulceroglandular, oculoglandular, oropharyngeal, pneumonic, and typhoidal syndromes (34).

Since *F. tularensis* is a gram-negative bacterium, it has a lipopolysaccharide (LPS) as a major component of its outer membrane. The LPS of *F. tularensis* is a smooth-type LPS (11). In several studies (2, 32), the carbohydrates of its O side chain have been shown to be important cell surface antigens that probably account for the specificity of the serological response.

Fulop et al. (10) have reported that the *F. tularensis* LPS exhibits as least one of the usual biological activities of LPSs: the ability to activate the complement system. However, several reports have shown that unlike LPSs from enterobacteria and many other gram-negative bacteria, the *F. tularensis* LPS does not act as a pyrogen (14), is not toxic for galactosamine-sensitized mice, has reduced activities in the *Limulus* amebocyte lysate assay, and induces low levels of tumor necrosis factor alpha (TNF-α) production and no interleukin 1 production in human monocytes (31).

The absence of certain in vitro cellular responses and in vivo host responses to this LPS may explain why *F. tularensis* can easily enter and replicate in macrophages, a phenomenon likely related to the high virulence of this organism (23). But the unusual properties of this particular LPS can also help to shed light on the mechanisms of cell activation by endotoxins. Indeed, LPSs from the large majority of gram-negative bacteria are known to interact and further activate a number of different cell types (4), and the mechanisms involved are still poorly understood. The aim of this investigation was to analyze a wide range of in vitro effects of the *F. tularensis* LPS in order to determine if this unusual LPS can be used as a tool to differentiate distinct pathways of LPS-induced activation of cells of the immune system.

MATERIALS AND METHODS

Mice. Swiss mice (5 to 7 weeks old) from R. Janvier (Le Genest Saint-Isle, France) and C3H/HeOU mice (8 to 10 weeks old) from the Breeding Center of the Pasteur Institute were used.

Media and reagents. Culture medium (CM) was RPMI 1640 (Gibco, Grand Island, N.Y.) containing 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 2-mercaptoethanol (5×10^{-5} M). Heparin, actinomycin D, crystal violet, and neutral red were purchased from Sigma Chemical Co. (St. Louis, Mo.). A solution of trypsin in EDTA was purchased from Biochrom KG (Berlin, Germany). Thioglycolate broth and minimal essential medium were from Diagnostic Pasteur (Ville d'Avray, France). Fluorescein isothiocyanate (FITC) was purchased from Sigma. The FITC-conjugated Fab fragment of a goat anti-mouse immunoglobulin M (IgM) monoclonal antibody was from Nordic (Tilburg, The Netherlands).

LPSs. The LPSs from *Salmonella choleraesuis* (LPS-Sc), *Salmonella typhimurium* LT2 (LPS-St), and *Bordetella pertussis* (phase 1 vaccine strain 1414) (LPS-Bp) were prepared by the phenol-water extraction procedure as described by Le Dur et al. (20). The LPS from *F. tularensis* LVS (LPS-Ft) was extracted and purified as described by Sandström et al. (31). Analysis of LPS-Ft by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed after silver staining a ladder pattern characteristic of a smooth-type LPS and after Coomassie brilliant blue staining an absence of protein bands, indicating a high level of purity.

LPS-Sc was labeled with FITC after CNBr activation and coupling to lysine (26). The method of activation of carbohydrates with CNBr (27) was used to activate LPS-Sc. The CNBr-activated LPS was then coupled to lysine by incubation for 150 min at 20°C, under gentle rotation, with lysine chloride (200

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µl, 5 mg/ml) in 1 M sodium hydrogen carbonate. The suspension of the lysine-LPS conjugate was dialyzed extensively against a 0.1 M sodium hydrogen carbonate buffer (pH 9). FITC (250 µl; 1 mg/ml in dimethyl sulfoxide) was added to the suspension of lysine-LPS (0.9 ml) in a plastic tube protected from light. The mixture was incubated for 150 min at 20°C under gentle rotation. After dialysis against phosphate-buffered saline (PBS), FITC-LPS was stored in the dark at −20°C.

Cells. Bone marrow cells (BMC) were collected from femurs of C3H/HeOU mice. Peritoneal macrophages were prepared from Swiss mice. Peritoneal exudates were harvested 5 days after intraperitoneal injection of 1.7 ml of thioglycolate broth by peritoneal washes with minimal essential medium containing 10 U of sodium heparinate per ml. Suspensions of peritoneal exudate cells (10^6 neutral red-positive cells per well) in RPMI buffered with 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid [HEPES]) were incubated (2 h, 37°C) in plates with 24 wells. Nonadherent cells were removed by four washings with minimal essential medium (500 µl per well).

The 70Z/3 pre-B-cell line was cultured (48 h, 37°C, 5% CO_2 in air; final density, 5×10^5 cells per ml) in CM supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS).

The murine fibroblast cell line L929-α was from M. Parant (Paris, France). Suspensions of L929 cells were prepared by treatment (1 min) of confluent cultures (75 cm²) with trypsin (2 ml, 0.05% in 0.02% EDTA), followed by addition of heat-inactivated horse serum (1 ml) to stop the reaction.

Flow cytometry. Stained cells (5,000 cells per sample) were analyzed on a fluorescence-activated cell sorting (FACS) flow cytometer (FACScan; Becton Dickinson Electronic Laboratories, Mountain View, Calif.) equipped with an argon ion laser and coupled to a microcomputer system (9153 B; Hewlett-Packard) running FACScan Research software. Cells were gated by uptake of propidium iodide to exclude dead cells (25) and by forward-angle light scatter to exclude small debris and large aggregates. Fluorescence histograms were generated with logarithmic amplification of light from single cells.

Estimation of TNF-α secretion. Macrophage culture supernatants were centrifuged (10 min, 900 × g) and rapidly assayed (without storage) for TNF-α activity by determination of the dilution that gave 50% killing of actinomycin D-sensitized L929 cells. Target cell viability was estimated by staining viable cells with crystal violet as described previously (19).

Nitrite determination. The concentration of NO_2^- in culture supernatants was used as an indicator of NO generated by nitric oxide synthase activity and was measured by the Griess reaction with sodium nitrite as a standard (5). Briefly, a solution of 1% sulfanilamide and 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4 was added to 100 µl in triplicate wells of a 96-well plate. The A_{550} was measured with a Dynatech plate reader.

Expression of sIg on pre-B cells. LPS-stimulated 70Z/3 cells (1×10^5 to 5×10^5) were incubated at 4°C for 20 min with the FITC-conjugated anti-IgM monoclonal antibody (final dilution, 1:200) in PBS (0.1 ml) containing 5% FCS and 0.07% sodium azide. The cell suspension was then centrifuged through a layer of 50% FCS in PBS. The pellets of FITC-labeled 70Z/3 cells were resuspended in 0.5 ml of PBS containing propidium iodide (0.2 µg/ml) to stain dead cells. Analysis of fluorescent 70Z/3 cells was done by flow cytometry. Viable cells with a fluorescence intensity higher than 6.4 (level of the highest autofluorescence intensity of unlabeled cells) were scored as expressing surface Ig (sIg).

Expression of inducible LPS receptors. BMC were incubated (24 h, 37°C) with the LPS. The stimulated cells (10^6 cells per ml) were stained by incubation (18 h, 4°C) with FITC-LPS (0.2 µg/ml) in CM. The cells were layered on a 50% FCS solution and centrifuged, and the cell pellet was resuspended in 0.5 ml of staining buffer (PBS, 5% FCS, 0.02% sodium azide) containing propidium iodide (0.2 µg/ml) to stain dead cells. Cells that expressed LPS-binding sites were detected by flow cytometry. Cells with a fluorescence intensity higher than the autofluorescence level (about 10 with the gain setting used) were scored as expressing the inducible LPS receptor.

RESULTS

TNF-α and NO production by mouse macrophages. In vitro, LPSs from gram-negative bacteria are potent stimulants of monocytes/macrophages (29). Activation of these cells with LPS usually elicits the production of different mediators, including TNF-α (35) and NO (15). However, it has been previously reported (31) that the *F. tularensis* LPS induces a low level of TNF-α in human monocytes. We analyzed the ability of LPS-Ft to induce TNF-α in mouse peritoneal macrophages. The levels of TNF-α secreted after exposure to LPS-Ft were compared with those induced by LPS-St and LPS-Bp, both of which are structurally characterized (3, 16) and able to activate mouse macrophages for TNF-α and NO production (9). The results in Fig. 1 are even more clear-cut than those previously found with human monocytes (31) and show that even at rel-
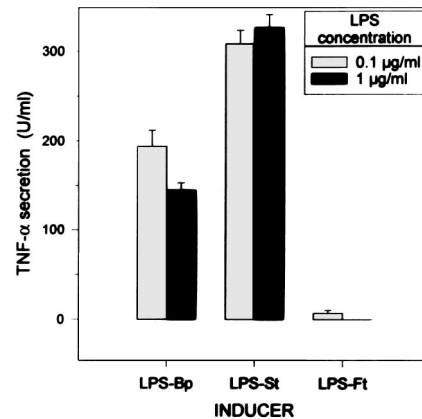

FIG. 1. LPS-induced secretion of TNF-α in mouse macrophages. The amounts of TNF-α in culture supernatants were estimated after incubation (18 h, 37°C) of thioglycolate-elicited peritoneal macrophages from Swiss mice (10^6 cells per well) with 0.1 and 1 µg of LPS-Bp, LPS-St, and LPS-Ft per ml. Data are means ± standard deviations of two determinations.

atively high doses (0.1 and 1 µg/ml), LPS-Ft fails to trigger TNF-α secretion in mouse peritoneal macrophages.

We also compared the levels of NO produced by these cells after exposure to the three LPSs. We found (Fig. 2) that the dose-response curves obtained with LPS-Bp and LPS-St were very similar, with an optimal level of NO produced in response to 100 ng of these LPSs per ml, whereas NO was undetectable after exposure to the same concentration of LPS-Ft. High doses of LPS-Ft (1 to 10 µg) induced NO production, but the concentration required to trigger the production of 50% of the maximal NO level was 1,000 times higher for LPS-Ft (1,000 ng/ml) than for the two standard LPSs (1 ng/ml).

Desensitization of mouse macrophages for endotoxin-induced TNF-α secretion. In addition to their large panel of direct biological activities, endotoxins can also induce a state refractory to their own effects, a phenomenon termed endotoxin tolerance, which can be observed during a second exposure to LPS (17). Tolerance to endotoxin acquired during tularemia has been reported by Greisman et al. (13). This finding could suggest that the *F. tularensis* LPS shares with
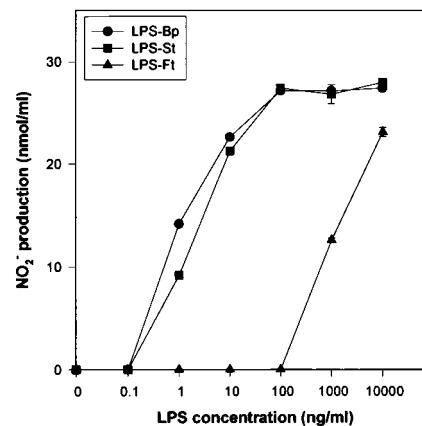

FIG. 2. LPS-induced production of NO in mouse macrophages. Peritoneal macrophages from Swiss mice (10^6 cells per well) were exposed for 24 h at 37°C to various concentrations of LPS-Bp, LPS-St, and LPS-Ft. Culture supernatants were harvested and assayed for NO_2^- accumulation. Data are means ± standard deviations of triplicate determinations.
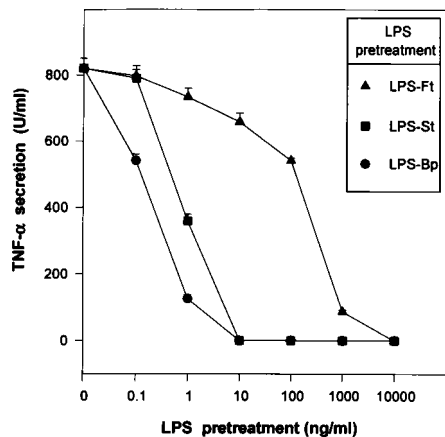
FIG. 3. LPS-induced desensitization of macrophages for TNF-α production. Thioglycolate-elicited peritoneal macrophages from Swiss mice (10^6 cells per well) were preincubated (18 h, 37°C) with various concentrations of LPS-Bp, LPS-St, and LPS-Ft in CM. The cells were washed three times (incubations for 40 min at 37°C in 0.5 ml CM) and exposed for 18 h at 37°C to LPS-St (10 μg/ml). Results are expressed as units of TNF-α produced after the second treatment and are means ± standard deviations of three values interpolated from serial dilutions of cell culture supernatants.

FIG. 4. LPS-induced priming of mouse macrophages for NO production. Thioglycolate-elicited peritoneal macrophages from Swiss mice (10^6 cells per well) pretreated (18 h, 37°C) with various concentrations of LPS-Bp (A) or LPS-Ft (B) were washed three times and exposed for 24 h at 37°C to 0.1 μg of LPS-St per ml or to medium alone. Culture supernatants were harvested and assayed for NO_2^- accumulation. Data are means ± standard deviations of triplicate determinations.

other LPSs the ability to induce endotoxin tolerance. Since this effect is due mainly to the desensitization of macrophages (38), it was of interest to reexamine this point in the simple in vitro model that we used in previous studies (8). We examined the abilities of LPS-Bp, LPS-St, and LPS-Ft to elicit in vitro, in mouse peritoneal macrophages, a reduction of the TNF-α response to a second contact with an active endotoxin. The results (Fig. 3) show that LPS-Bp and LPS-St have similar activities, whereas LPS-Ft is considerably less active: a 50% inhibition of TNF-α secretion required 0.2 ng of LPS-Bp, 1 ng of LPS-St, and 200 ng of LPS-Ft per ml.

Priming of mouse macrophages for enhanced NO responses to endotoxin. It has been reported recently (7, 40) that preexposure to LPS, which induces reduced cytokine responses to LPS challenge, simultaneously primes the macrophages for an enhanced NO response to LPS restimulation. We compared the abilities of LPS-Bp and LPS-Ft to induce this priming effect for NO responses to 0.1 μg of LPS-St per ml. We observed that macrophages pretreated with LPS-Bp, washed, and reincubated in CM alone produced NO, as a consequence of residual NO synthase activity (Fig. 4A), whereas macrophages pretreated with LPS-Ft did not (Fig. 4B). This finding is in line with the direct effects of these LPSs on NO production shown in Fig. 2. NO was also produced when the cells were exposed to LPS-St (0.1 μg/ml) during the second incubation (Fig. 4). This secondary NO response to LPS can be estimated by the difference between levels of NO production in cells reincubated with LPS and CM alone. The results show that this difference (4 nmol/ml) increased upon pretreatment of the cells with 0.1 to 1 ng of LPS-Bp per ml (Fig. 4A) but did not increase in LPS-Ft-pretreated cells (Fig. 4B). This result shows that LPS-Bp can prime macrophages for an enhanced NO response to an LPS challenge, whereas LPS-Ft cannot.

LPS-Ft fails to block endotoxin-induced effects in macrophages. The results mentioned above show that several effects that usually follow the interaction of standard LPSs with macrophages are not triggered by LPS-Ft. We wished then to determine whether the presence of LPS-Ft can antagonize the effects of an active LPS in macrophages. We examined the influence of the presence of LPS-Ft on LPS-Bp-induced acti-
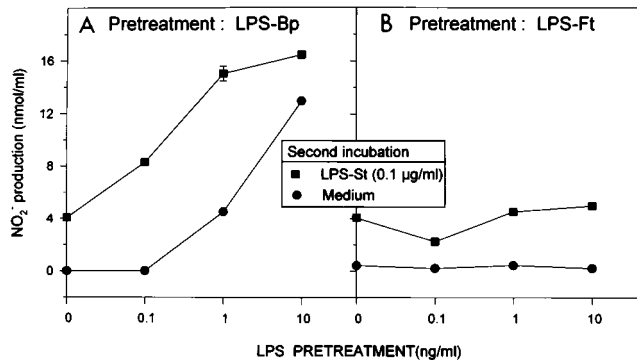
vation of macrophages for TNF-α and NO production (Fig. 5A and B) and on LPS-Bp-induced desensitization of the cells for TNF-α secretion (Fig. 5C). The results show that the three activities of LPS-Bp observed in the absence of LPS-Ft (Fig. 5, left histograms) are not blocked or reduced by the presence of 10 or 100 ng of LPS-Ft per ml, added to CM 1 h before LPS-Bp (Fig. 5, middle and right histograms).

Induction of sIg expression on pre-B lymphocytes. Another well-known effect of endotoxins is their ability to activate lymphocytes of the B lineage. To study this effect, the 70Z/3
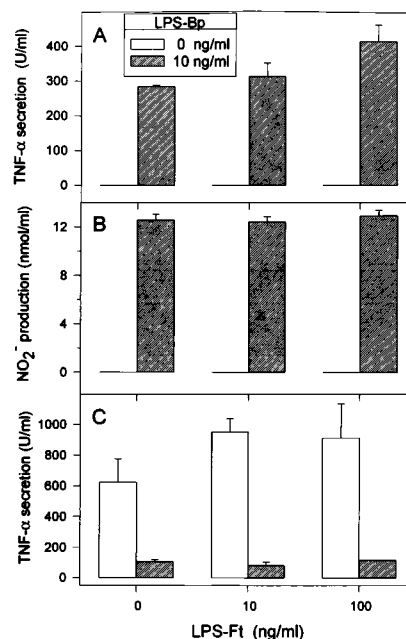

FIG. 5. Influence of LPS-Ft on LPS-Bp-induced effects in mouse macrophages. Thioglycolate-elicited peritoneal macrophages from Swiss mice (10^6 cells per well) preexposed (1 h, 37°C) to different concentrations of LPS-Ft were further incubated (18 h, 37°C) with 10 ng of LPS-Bp per ml or with medium alone. Culture supernatants were harvested and assayed for the presence of TNF-α (A) and NO_2^- (B). In a third experiment designed to analyze LPS-Bp-induced desensitization (C), cells treated as described above were washed three times, and their TNF-α response to 0.1 μg of LPS-St per ml was determined. Data are means ± standard deviations of two determinations.
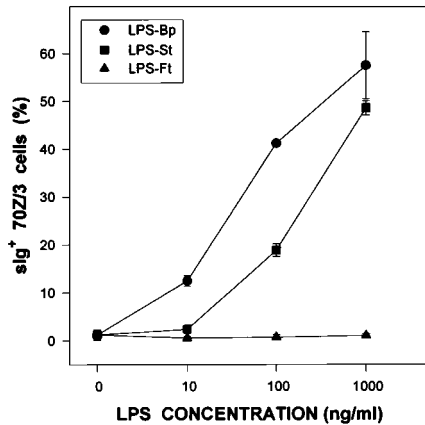
FIG. 6. LPS-induced expression of sIg on pre-B cells. 70Z/3 cells (5×10^4 cells per well) were incubated (48 h, 37°C) with various concentrations of LPS-Bp, LPS-St, and LPS-Ft in CM. The cells were stained with an FITC-conjugated Fab fragment of a goat anti-mouse IgM monoclonal antibody. The percentage of viable cells with a fluorescence intensity higher than the autofluorescence level was determined by FACS analysis. Data are the means ± standard deviations of two determinations.
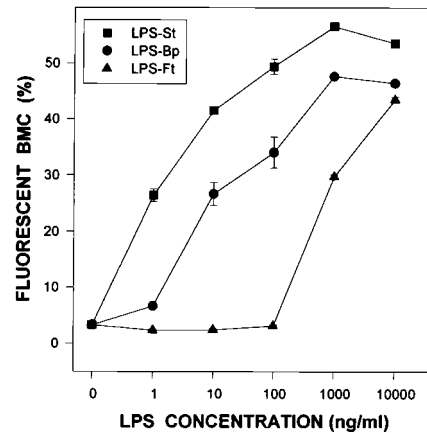

FIG. 7. Induction of LPS receptors on BMC. BMC were incubated at 37°C for 24 h with various concentrations of LPS-Bp, LPS-St, and LPS-Ft in CM. The stimulated cells were washed three times, equilibrated for 1 h at 4°C in CM, and incubated (18 h, 4°C) with FITC-LPS (1 μg/ml). The percentage of fluorescent cells was determined by FACS analysis. Data are means ± standard deviations of two determinations.

murine pre-B-cell line is a suitable and well-established model. The cells produce μ mRNA in the absence of stimulation, and LPS triggers the synthesis of κ mRNA, leading to the expression of sIgM (24). We compared LPS-Ft and the two standard LPSs for their abilities to induce sIg expression on 70Z/3 pre-B cells. The cells were incubated for 48 h at 37°C with various concentrations of the LPSs, and the expression of sIg was analyzed by flow cytometry after staining with a fluorescent anti-IgM monoclonal antibody. The results in Fig. 6 show dose-dependent activities of LPS-Bp and LPS-St, whereas LPS-Ft was inactive, even at the highest dose used (1 μg/ml).

Induction of LPS receptor expression in BMC. In addition to lymphocytes of the B lineage and macrophages, bone marrow granulocytes represent a third LPS-responsive cell type. We have shown previously (12, 26) that unstimulated mouse BMC do not bind FITC-LPS but express specific binding sites for this ligand after exposure to nanomolar concentrations of LPS. Therefore, we compared the abilities of LPS-Bp, LPS-St, and LPS-Ft to induce the expression of LPS-binding sites on mouse BMC. The cells were incubated for 24 h at 37°C with various concentrations of LPS (1 ng to 10 μg), washed to remove LPS, stained with FITC-LPS, and analyzed by flow cytometry. We found (Fig. 7) that LPS-St was the most efficient inducer of LPS-binding sites. With LPS-Bp and LPS-Ft, higher concentrations (10 and 1,000 times, respectively) were required for the activation of the same percentage of BMC.

Interaction with the inducible LPS receptor of BMC. It was also of interest to determine whether LPS-Ft can interact with the newly expressed receptor induced by an active LPS. The expression of the inducible receptors was first triggered by incubation of mouse BMC for 24 h at 37°C with 0.1 μg of LPS-Bp per ml. The interaction of LPSs with these inducible LPS-binding sites was then analyzed by estimation of their abilities to inhibit the binding of FITC-LPS to the stimulated cells. LPS-Ft was compared by this method with LPS-Sc. We found that preincubation of activated BMC with increasing concentrations of LPS-Sc (0, 0.2, 2, and 20 μg/ml) reduced the percentage of cells able to bind FITC-LPS (21.9, 19.6, 12.1, and 8.8%, respectively), whereas preincubation with LPS-Ft at the same concentrations did not reduce the percentage of such cells (21.9, 21.7, 23.9, and 21.3%, respectively). Therefore,

LPS-Sc can be considered a good inhibitor of the binding of FITC-LPS to the inducible receptors (60% inhibition with 20 μg of LPS-Sc per ml), whereas LPS-Ft was completely inactive.

DISCUSSION

We compared LPS-Ft with two LPSs of known chemical structures and of conventional biological activities, belonging to different genera: the *S. typhimurium* smooth-type LPS, which contains a long carbohydrate chain with repeating units, and the *B. pertussis* LPS, with a short carbohydrate moiety (dodecasaccharide) devoid of repeating units. We examined first the ability of these LPSs to induce direct activation of three different cell types: peritoneal macrophages for the production of TNF-α and NO, pre-B cells for the expression of sIg, and BMC for the expression of LPS-binding sites. Our study shows that in all of these tests (Fig. 1, 2, 6, and 7), activation with LPS-Ft either was undetectable or required concentrations 100 to 1,000 times higher than for standard LPSs. The inability of LPS-Ft to activate cells of the immune system may be beneficial to the microorganism, particularly as regards the lack of LPS-induced production of NO, which is usually a very efficient antimicrobial mechanism mediated by activated macrophages.

Although incubation with LPS-Ft did not induce direct activation of cells, it was of interest to examine the influence of this incubation on a second challenge with an active endotoxin, since LPS pretreatment usually induces an increase of secondary NO responses (40) and a decrease of secondary TNF-α responses (endotoxin tolerance) (38). An old observation showing that human volunteers convalescing from induced tularemia acquired tolerance to the pyrogenic action of endotoxin (13) prompted us to examine whether LPS-Ft, although unable to induce direct cell activation, could up- or down-regulate secondary LPS responses. We found that preexposure to LPS-Ft did not trigger down-regulation of TNF-α (Fig. 3) or up-regulation of NO (Fig. 4) responses to an endotoxin challenge. The tolerance effect acquired during tularemia that was reported by Greisman et al. (13) may thus be due to other constituents of the *F. tularensis* microorganism or, more likely, to mediators produced in vivo during infection with this pathogen. For instance, it has been established that glucocorticoids

(33), cytokines (interleukin 1α and TNF-α) (6, 39), and growth factors (transforming growth factor β) (36) can also induce endotoxin tolerance.

The inability of LPS-Ft to directly activate different cell types or to modulate their responses to a second exposure to endotoxin could be due either to a total absence of interaction with LPS receptors or to interactions that do not trigger receptor-mediated signalling. In the latter case, LPS-Ft could block the binding between standard LPSs and their receptors and thus behave as an LPS antagonist. Our experiments designed to test this hypothesis showed, however, that LPS-Ft did not inhibit the binding of FITC-LPS to the inducible LPS receptor of BMC and did not antagonize TNF-α and NO production (Fig. 5A and B) and cell desensitization (Fig. 5C) induced by a standard LPS in macrophages. We can thus conclude that the inability of LPS-Ft to induce the usual panel of cell responses to endotoxin is likely due to the failure of LPS receptors to interact with LPS-Ft.

As already observed with other LPSs which exhibit atypical biological activities, such as those of *Rhodobacter sphaeroides* (28), *Rhodobacter capsulatus* (18), and *Helicobacter pylori* (22), the absence of activity of LPS-Ft on various cell types can probably be ascribed to some unusual structural feature in its lipid A region. Very few studies have been carried out on the structure of LPS-Ft. It is noteworthy that *F. tularensis* cannot be serotyped because all isolated strains were antigenically similar. The carbohydrate region of its LPS is one of the major antigens of this microorganism (2, 32), which may indicate that all *F. tularensis* strains have the same O-specific antigen. The O-specific polysaccharide isolated from the LPS of a vaccine strain of *F. tularensis*, derived from the same as that used in our study, has been analyzed by Vinogradov et al. (37). It has structural similarities with O-specific polysaccharides of biologically active LPSs such as those of *Pseudomonas aeruginosa* O6 and *Shigella dysenteriae* type 7. In contrast, very little information is available on the structure of the lipid A region of LPS-Ft. In view of the absence of activity reported in our study, a detailed structural analysis of this region is warranted.

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