

Original Research

Effect of Endotoxins Isolated from *Desulfovibrio desulfuricans* Soil and Intestinal Strain on the Secretion of TNF- α by Human Mononuclear Cells

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Abstract

Mononuclear cells play an important role in the regulation of microbe-induced inflammation, in part through their ability to secrete cytokines in response to microorganisms and their products. To evaluate the effects of *Desulfovibrio desulfuricans*-derived endotoxins on TNF- α induction, lipopolysaccharides (LPSs) isolated from soil and intestinal strain were used to stimulate peripheral blood mononuclear cells. The effect of these LPSs was assessed in comparison to that of LPSs from *Escherichia coli*, *Salmonella minnesota* and of lipid A from *Salmonella minnesota*. Level of TNF- α was measured by enzyme-linked immunosorbent assay. *D. desulfuricans* LPSs at the highest dose (1000 ng/ml) displayed greater biological potency in inducing TNF- α secretion than other endotoxins used which indicates that these LPSs may act as a critical regulatory factor in bacteremia caused by these microorganisms.

Keywords: mononuclear cells, TNF- α , lipopolysaccharides, *Desulfovibrio desulfuricans*

Introduction

Bacteria of the *Desulfovibrio desulfuricans* species are Gram-negative anaerobic rods selectively reducing sulphates and colonizing oxygen-free ecosystems. They have been isolated from most environmental sources, such as sewage, industrial effluents, and most types of water and soil [1, 2]. Sulphate-reducing bacteria participate in the degradation of organic matter in the natural environment [3] and play an important role in the removal of heavy metals, such as mercury, chromium, and copper from various environments [4]. The growing interest in these bacteria arose from the 1970s, when their presence in human feces was demonstrated by Beerens and Romond [5]. It

has been suggested that under certain conditions *D. desulfuricans* bacteria can become etiologic factor of various types of enteritis [6, 7]. They have been found to reside in increased population in the digestive tract of patients suffering from ulcerative colitis and Crohn's disease [8, 9]. In addition, cases of bacteremia, bacteriuria, liver abscess and septicemia caused by these bacteria have been described [10-13].

Like the cell envelope of all other Gram-negative bacteria, that of *D. desulfuricans* contains lipopolysaccharide (LPS). In general, LPS is composed of polysaccharide subdivided into the O-specific chain (O-antigen) and the core oligosaccharide which is covalently bound to the lipid portion, termed lipid A [14]. LPS is essential for bacterial growth and viability. However, when released from the bacterial surface, LPS plays an important role in the

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pathogenesis and manifestation of Gram-negative infection in general, and of septic shock in particular, and is thus called endotoxin [15]. It has been established both *in vitro* and *in vivo* that endotoxin has the ability to activate host cells, especially blood mononuclear cells which are the principal cell types regulating local inflammatory responses to bacterial insult by the production of cytokines [16, 17]. Stimulation of mononuclear cells by LPS was suggested to activate the initial steps of the immune response with the involvement of macrophages and granulocytes, while infection with live bacteria could also stimulate the latter phase of the immune response, probably due to the effect of initially produced cytokines [18].

Among the variety of up-regulated and secreted cytokines, the key role in orchestrating the complex events involved in inflammation and immunity seen in sepsis has been ascribed to tumor necrosis factor- α (TNF- α) [19]. The finding that in mononuclear cells stimulated by LPS, TNF- α was produced in higher quantities than other cytokines indicates a greater role of TNF- α in inducing bacterial fever [18]. Using the whole-blood model, Nancy et al. [20] confirmed the results of other earlier published study performed with isolated mononuclear cells [21], where increased production of TNF- α upon bacterial endotoxin stimulation was observed in healthy subjects and this LPS-induced production could be reduced by butyrate, a normal metabolic product of intestinal bacterial flora. Studies by Ulmer et al. [22] established that T-lymphocytes could contribute to an inflammatory reaction to LPS, which is a potent inducer of these cells' proliferation and cytokine production. CD14 was postulated to be a pattern recognition factor that recognizes a wide variety of bacterial products, especially LPS, leading to cell activation [23, 24]. Recently, Toll-like receptor-4 (TLR4) was found to mediate the intracellular signaling after LPS binding to CD14 [25]. High LPS concentrations induce TNF- α production via a CD14-independent pathway [26].

TNF- α is expressed by monocytes as a transmembrane protein which is proteolytically cleaved by TNF- α -cleaving enzyme (TACE) upon cellular activation by LPS or live bacteria [27]. This can explain the rapid release of TNF- α by monocytes in whole blood after 20 min of LPS incubation, as observed by van Leeuwen et al. [28] and after 3 hours following whole blood stimulation with LPS in *in vitro* studies by Allen et al. [29]. In other studies, the immediate release of TNF- α by peripheral blood mononuclear cells in response to LPS stimulation remained stable during 24 hours [18]. The TNF- α level in blood plasma can reflect the toxicity of LPS and high levels of TNF- α correlate with increased risk of mortality [30, 31]. The biological activity of endotoxins may, thus, be discussed in the context of the ability to induce TNF- α synthesis/secretion by cells exposed to them. The activity of endotoxins can vary depending on the bacterial strains and types, endotoxin concentration, duration of host tissues contact with LPS. Additionally, differences of LPS chemical structure can influence their physical aggregate structures and their activity [32].

The biological significance of endotoxin release in the complex pathology of *D. desulfuricans* infections has not much been explored. Our previous studies demonstrated that LPS derived from the soil and intestinal strain of these bacteria were not a stimulus for interleukin-8 (IL-8) secretion by human intestinal epithelial cells [33]. On the contrary, these endotoxins activated endothelial cells to express surface adhesion molecules and increase IL-8 and IL-6 secretion [34]. To further explore the activity of *D. desulfuricans* LPS as a potential factor in bacterial pathogenesis, we examined whether this LPS is functionally different from other Gram-negative bacteria endotoxins in its ability to activate the mononuclear cells of whole blood. For that, we assessed the effect of LPS from *D. desulfuricans* in comparison to that of LPSs from *Escherichia coli* and *Salmonella minnesota* and that of lipid A from *S. minnesota* on TNF- α secretion by human peripheral blood mononuclear cells.

Materials and Methods

Bacterial Strains and the Isolation of Bacterial Endotoxins

The wild strain DV-A/94 of *D. desulfuricans* was isolated from feces derived from patient suffering from siderotic anaemia and cholestasis by the method described previously [35]. The *D. desulfuricans* soil type strain La 2226 (DMS642) was purchased from the Swiss National Collection of Type Cultures, Lausanne, Switzerland. Bacteria of both strains were cultured anaerobically in Postgate's liquid medium B containing lactate and sulphate [36]. Endotoxic LPSs were isolated from bacteria in accordance with the hot phenol-water method of Westphal et al. [37]. Briefly, a bacterial pellet was digested with lysosyme (Sigma) and the cell lysate was incubated with DNase and RNase to digest nucleic acids. Then, 90% phenol extraction procedure was used. The phenol layer was rejected and the water one was dialyzed against deionized water, then digested with proteinase K and dialyzed again. Finally, LPSs were lyophilized using Labconco equipment (Freeze Dry System/Freezone 4.5).

The biological effectiveness of isolated LPSs was tested in a *Limulus* Amebocyte Lysate (LAL) assay according to the manufacturer's instructions (BioWhittaker, Inc.) [38].

In the experiment, LPSs from *E. coli* 0111:B4 and *S. minnesota* Re595 and lipid A from *S. minnesota* Re595 were used as references. They were obtained from Sigma.

Preparation of LPS and Lipid A Concentrations

Stock solutions of 1 mg/ml of each LPS and lipid A were made in RPMI 1640 culture medium (Sigma) and

vortexed prior to use for 10 min using ultrasonic bath (Decon Ultrasonic Ltd., England). Dilutions of 0.5, 2.5, 5, 10, 50, 500, 1000 ng/ml in RPMI 1640 medium were used in the study.

Isolation of Human Mononuclear Cells

Mononuclear cells were isolated from peripheral blood obtained from healthy human volunteers after informed consent. Blood was collected into heparin tubes and diluted 1:1 with phosphate-buffered saline (PBS), pH 7. Cell isolation was carried out according to a modified Boyüm [39] procedure based on Gradi-sol L (density 1.077 g/ml; Polfa, Poland) gradient. Gradi-sol L was stratified on a whole diluted blood. After centrifugation for 15 min at 600g at 4°C, three layers were received and the central one was transferred to another tube. This fraction was washed with PBS three times and then, with RPMI 1640 medium once. Culture medium was rejected and the cell pellet was resuspended in 1 ml of RPMI medium. Cells were counted under a light microscope using direct Bürker method with the application of Türk liquid chemical (lysis of erythrocytes). The viability of the cells, judged by erythro-zine exclusion, exceeded 95%.

Cell Culture and Treatment

The cells (10^6 /ml) were cultured in 25-cm² tissue culture flasks (Nunc) in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum (BIOCHROM KG; tested for mycoplasma), 50 µl of β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma). Cells were incubated at 37°C in humidified atmosphere containing 5% CO₂, alone or with concentrations of stimuli indicated above, which were added in 10 µl volume into the cultures. The stimulation lasted 24 h and then cell culture supernatants were collected, cleared by centrifugation and frozen at -20°C until assayed for TNF-α.

Assay of TNF-α Secretion

The amount of TNF-α secreted into the culture supernatants was determined by an enzyme-linked immunosorbent assay (ELISA; Quantikine R&D Systems). The sensitivity limit of the assay was less than 4.4 pg/ml. The content of TNF-α was measured within each supernatant in triplicate. Colorimetric results were read on a Elx800 96-well plate reader (BIOTEK Instruments Inc.) at a wavelength of 450 nm. The concentrations of TNF-α were determined by comparison with the TNF-α standard curve, which ranged from 0-1000 pg/ml.

Statistical Analysis

The comparison of TNF-α concentrations in culture supernatants evoked by different concentrations of the same LPS or lipid A was analyzed by one-way ANOVA (p values are presented in Table 1). One-way ANOVA was also used to compare TNF-α contents between supernatants derived from cell cultures exposed to various LPS/lipid A of the same concentrations (p values are presented in Table 1). Since the hypothesis "Ho" referring to equality of all group means was rejected, the means were compared in detail using the Tukey HSD test and the results of this analysis (p values) are presented in the Result section. All the results were expressed as means ± SD of three independent experiments, and p < 0.05 was considered statistically significant. Statistical analysis was performed using the Statistica PL. V 6.0. computer program.

Results

Determination by the LAL test confirmed the ability of endotoxins isolated from the intestinal and soil strain of *D. desulfuricans* to stimulate the *Limulus* Amebocyte Lysate (LAL) cascade.

Analysis of the TNF-α secretion by human mononuclear cells showed that in the absence of bacterial LPS these cells did not release any detectable TNF-α. They responded, however, to bacterial products activation by secreting TNF-α. The TNF-α concentration dependency of LPS/lipid A dose for the studied bacteria species is shown in Table 1. The results are expressed as mean cytokine levels ± the standard error of the mean of triplicate samples and are representative of three separate experiments.

As shown in Table 1, the release of TNF-α from the cells was bacterial stimuli dose-dependent. A comparison of TNF-α secretion levels in the cell cultures treated with different concentrations of the same LPS or lipid A revealed significant differences for all investigated endotoxins (one-way ANOVA). Lower doses of LPSs/lipid A (0.5-5 ng/ml) had relatively little effect on the cells, manifested by TNF-α levels secreted in the range of 47.53 ± 4.65 to 377.45 ± 29.65 pg/ml. However, the statistical analysis showed insignificant differences between TNF-α amounts secreted following stimulation with 0.5, 2.5 and 5 ng/ml of the same endotoxin (p=0.9959-1.0, Tukey test). Higher concentrations of LPSs/lipid A (10-1000 ng/ml) stimulated markedly greater response of the cells, and the TNF-α secretion achieved the levels of 1407.70 ± 70 to 12775.50 ± 2493.50 pg/ml (Table 1). In comparison to the doses of 0.5, 2.5 and 5 ng/ml of LPSs and lipid A, their concentration of 50 ng/ml appeared to be highly cytokine stimulatory, as indicated by statistical significance, respectively, as follows: for LPS from *D. desulfuricans* soil strain, p=0.0002, p=0.0002, p=0.0002 (Tukey test); for LPS from *D. desulfuricans* intestinal strain, p=0.0185, p=0.0192, p=0.0211 (Tukey test); for LPS from *E. coli*, p=0.0009, p=0.0010, p=0.0011 (Tukey test); for LPS

from *S. minnesota*, $p=0.0045$, $p=0.0048$, $p=0.0052$ (Tukey test); for lipid A from *S. minnesota*, $p=0.0075$, $p=0.0109$, $p=0.0154$ (Tukey test).

Treatment of cells with the highest dose (1000 ng/ml) of LPS from *E. coli* resulted in a decrease in the cytokine secretion compared to the effect of the cells stimulation with this LPS at 500 ng/ml (Table 1). Statistical analysis revealed the considered difference in the TNF- α amounts released by cells to be insignificant ($p=0.9869$, Tukey test). This indicates that the ability of *E. coli* endotoxin to induce TNF- α production reached a plateau at a dose of 500 ng/ml. Treatment of cultures with LPS from *S. minnesota* and with lipid A at the concentration of 1000 ng/ml induced insignificantly higher levels of TNF- α secretion than those seen in cultures treated with these endotoxins at 500 ng/ml dose ($p=0.9995$ for LPS from *S. minnesota*, and $p=0.4176$ for lipid A, Tukey test).

These observations were in contrast to the effect of endotoxins derived from both strains of *D. desulfuricans*, which at a dose of 1000 ng/ml caused a statistically significant increase in TNF- α release from cells (Table 1) in comparison to the level of TNF- α produced by their lower dose (500 ng/ml) (for soil strain LPS, $p=0.0003$ and for intestinal strain LPS, $p=0.0062$, Tukey test). This indicates that *D. desulfuricans* LPSs differed from other bacteria' stimuli used in this study, in the biological potency *in vitro*.

The content of TNF- α in cell cultures exposed to the same concentrations within the range of 0.5-500 ng/ml of different LPSs and lipid A did not show statistically significant differences (one-way ANOVA; Table 1). On the contrary, when endotoxins were added to the cultures at the concentration of 1000 ng/ml, the cellular response in terms of TNF- α amount secreted was differentiated depending on endotoxin origin ($p=0.0107$, one-way ANOVA, Table 1). The most potent stimulators appeared to be

LPSs from *D. desulfuricans* both soil and intestinal strain, which induced significantly more TNF- α (11947.50 ± 1426.50 and 12755.50 ± 2493.50 pg/ml, respectively) than the same dose of LPSs from *E. coli* and *S. minnesota*, and of lipid A. The stimulatory effects of both strains of *D. desulfuricans* were similar in potency, as manifested by statistically insignificant difference ($p=0.9797$, Tukey test) in the concentration of TNF- α secreted.

Discussion

Very little is currently known about the role that *D. desulfuricans* may play within the digestive tract and about the immunologic potential of LPS derived from these microorganisms. Reported cases of bacteremia caused by these bacteria suggest a possible role of their certain components in the pathology associated with infections mediated by these microorganisms. The increased permeability of the intestinal mucosa associated with some intestinal disorders as well as various medical and surgical treatments may be a major cause of translocation of microbial flora, including *D. desulfuricans* into the blood [40]. However, how these bacteria get into the organism from the natural environment remains uncertain.

The present study investigated whether *D. desulfuricans* LPS differs from *E. coli* and *S. minnesota* LPSs and from lipid A in the ability to induce TNF- α secretion by mononuclear cells. The interaction between LPSs and these cells has been studied at a wide range of LPS concentrations, including those that have been described as being present in the blood of patients with Gram-negative sepsis [41]. Troelstra et al. [42] studied the interaction of *S. minnesota* fluorescein-labeled LPS (FITC-LPS) with monocytes in a broader range of LPS concentrations, up to 2500 ng/ml and found that the binding of FITC-LPS

Table 1. Dose response of TNF- α secretion by whole blood mononuclear cells stimulated with LPS from *D. desulfuricans* intestinal and soil strain, LPS from *E. coli* 0111:B4, *S. minnesota* Re595, and with lipid A from *S. minnesota* Re595.

LPS/lipid A concentration [ng/ml]	TNF- α [pg/ml]					One-way ANOVA p
	LPS from <i>D. desulfuricans</i> intestinal strain	LPS from <i>D. desulfuricans</i> soli strain	LPS from <i>E. coli</i> 0111:B4	LPS from <i>S. minnesota</i> Re595	Lipid A from <i>S. minnesota</i> Re595	
0.5	282.27 \pm 24.68	48.93 \pm 5.78	65.90 \pm 10.64	47.53 \pm 4.65	58.90 \pm 5.89	0.185
2.5	304.93 \pm 26.70	190.37 \pm 27.37	98.57 \pm 15.83	88.17 \pm 11.59	225.00 \pm 22.50	0.722
5	358.30 \pm 28.32	314.73 \pm 33.24	197.07 \pm 26.92	134.57 \pm 21.59	377.45 \pm 29.65	0.752
10	2059.83 \pm 670.60	2331.33 \pm 408.25	2102.00 \pm 616.94	1628.53 \pm 130.83	1407.70 \pm 703.30	0.638
50	4160.67 \pm 752.10	5548.00 \pm 730.43	5829.33 \pm 840.40	4763.67 \pm 873.48	3394.50 \pm 850.50	0.437
500	8243.50 \pm 982.45	8732.33 \pm 802.23	9584.44 \pm 902.07	7754.33 \pm 740.92	5382.50 \pm 936.50	0.240
1000	12755.50 \pm 2493.50	11947.50 \pm 1426.50	8883.50 \pm 835.50	8132.00 \pm 654.00	6876.00 \pm 1020.16	0.0107
One-way ANOVA p	0.0064	0.0020	0.0121	0.0132	0.0325	

to these cells was CD14-dependent at up to 100 ng/ml of LPS per ml. Several authors described unsaturable binding of FITC-LPS to monocytes, i.e., at 1 to 100 µg of FITC-LPS per ml [43] and at up to 50 µg of FITC-LPS per ml [44]; one study showed saturation at 1 µg/ml [45]. Unsaturable binding was also described for human peripheral blood monocytes incubated with concentrations of up to 10 µg of ¹²⁵I-LPS per ml [46]. According to the studies by Karahashi et al. [32], the wild type LPSs from *E. coli* and *S. minnesota* exhibited the highest activity at the concentrations exceeding 10 ng/ml in the stimulating TNF-α production by macrophages.

In the present study all examined endotoxins evoked a weak response of mononuclear cells at doses below 10 ng/ml. At the range of 10-50 ng/ml, these endotoxins were capable of significant activation of the target cells, whereas larger amounts of the stimuli induced significantly higher levels of TNF-α secreted. Considering responsiveness to LPSs, a striking difference was seen between LPSs derived from both strains of *D. desulfuricans* and other bacteria'endotoxins at their highest concentration (1000 ng/ml) used. LPSs from *D. desulfuricans* displayed an enhanced ability to induce TNF-α secretion compared to *S. minnesota* and *E. coli* LPSs as well as to lipid A, for which the plateau effect was observed at a dose of 500 ng/ml and they did not promote greater TNF-α release at higher concentration. This finding may indicate that *D. desulfuricans* LPS is capable of inducing greater immune response than *E. coli* and *S. minnesota* LPS because at twice higher dose (1000 ng/ml) the increase in TNF-α release was still observed. It can be suggested that increased expression of LPS receptors on the mononuclear cells may account for their enhanced binding ability to produce TNF-α when treated with increasing doses, such as 1000 ng/ml of *D. desulfuricans* LPS. As more receptors are available on the cell surface, the cytokine response to the LPS may be further enhanced. Moreover, the binding of *D. desulfuricans* LPS can also be CD14-independent process, which according to the literature data may not be saturable at all, the more LPS is added, the more association occurs [44]. Besides, Troelstra et al. [42] observed that the association of LPS from *S. minnesota* Re595 with monocytes was a saturable phenomenon to the extent that CD14 is involved.

Lipid A from *S. minnesota* used in this study stimulated a weaker response of the cells by inducing relatively less TNF-α than the corresponding doses of all bacterial LPSs. It has been established that lipid A is essential for the endotoxic effects of Gram-negative bacteria *in vitro* and *in vivo*. The structural features of this region determine the biological activities of LPS complexes. Studies with chemically defined synthetic analogs and partial structures of *E. coli* and *S. minnesota* species lipid A have provided clear evidence that slight modifications to lipid A architecture (e.g., the loss of one constituent from the molecule or a different distribution of constituents) result in a significant reduction in biological activity [14]. Hexaacetylated *E. coli* lipid A has the highest cytokine-in-

ducing capacity, whereas lipid A with four fatty acids is endotoxically inactive but expresses antagonistic activity against active LPS [47]. Studies by Mueller et al. [47] also demonstrated that LPS-binding protein (LBP) which is involved in the activation of mononuclear cells by LPS was a target for the inhibitory function of *E. coli* lipid A with four fatty acids which strongly inhibited LPS-induced TNF-α release. Partial structures of LPS were also reported to reduce cytokine production from mononuclear cells induced by peptidoglycan and LPS [48]. LPS of Re595 mutant of *S. minnesota* used in this study, lacks O antigen and part of the core polysaccharide but it has 2-keto-3-deoxyoctane (KDO) left on lipid A and this could explain its lower capacity in the TNF-α induction from mononuclear cells compared to *E. coli* and *D. desulfuricans* LPS. Re595 lipid A used in the present study differs in structure from Re595 LPS by lacking KDO, which is known to be required for expression of some activities by lipid A. The lower activity of *S. minnesota* lipid A compared to *S. minnesota* LPS, as observed in our study, can therefore, result from the lack of KDO in lipid A. However, when the effects of these two preparations on TNF-α induction in murine macrophage cell line RAW 264.7 were compared, the responses of cells were almost equal [49]. Based on the literature data provided in this paper, concerning structure-activity relationship of lipid A, and its lower activity compared to all LPS used, as observed in this study, it can be concluded that lipid A determines the endotoxic effects of Gram-negative bacteria when it is a part of LPS complex. As suggested by Muotiala et al. [50], the primary role of lipid A should not be considered endotoxicity alone, but that it can provide a functional macromolecular matrix through which the bacterium interacts with its environment.

Chemical structure of LPS from *D. desulfuricans* has not been determined, as yet. Gaylarde et al. [51] made an attempt to establish the sugar profile of these endotoxins. They found rhamnose, glucose, galactose, mannose, ribose in the carbohydrate component and suggested the studied endotoxins to be devoid of KDO. Lodowska et al. [52] identified all the above sugars except ribose in the oligosaccharide component and demonstrated the presence of KDO in LPS isolated from *D. desulfuricans*.

Although significant differences in LPS responses are apparent between closely related species, and there are substantial differences even among genetically heterogeneous members of the same species [53], LPSs isolated from intestinal and soil strain of *D. desulfuricans* showed similar potency in stimulating mononuclear cells to TNF-α secretion in the present study. This finding agrees with our previous observations showing similar activity of LPSs isolated from both strains in regard to colonocytes and endothelial cells stimulation [32, 33]. The comparable biological activity demonstrated by the two studied *D. desulfuricans* strains in relation to intestinal, endothelial as well as mononuclear cells in terms of their secretion of cytokines, cannot, however, be explained by the genetic relationship of two isolates. Our genotypic studies on the intraspecies variability

of the two *D. desulfuricans* strains, as analyzed by genomic profiles using PCR-based methods (REP-PCR, ERIC-PCR, AP-PCR), revealed distinct DNA fingerprint patterns of two isolates indicating a relatively low level of similarity [54, 55]. On the other hand, the analysis of the cellular fatty acid profiles, generally accepted by taxonomists as chemotaxonomic marker [56] for the two investigated strains revealed sufficiently similar patterns confirming the identification of these strains as members of *D. desulfuricans*. Moreover, intestinal strain was characteristic of higher ratio of saturated to unsaturated fatty acids than did soil strain and both strains could also be distinguished by the relative amounts of i-C_{17:1} and i-C_{15:0} as key branched-chain fatty acids [57]. Both *D. desulfuricans* strains appeared, however, to be similar in respect to susceptibility to antibiotics and they were practically identical in respect of their biochemical properties related to the capability of fermentation of carbohydrates, production of hydrogen sulphide, indole and β -lactamases, and the presence of several enzymatic activities [58].

The findings of the present work indicate that *D. desulfuricans* LPS can be an important virulence factor that plays a role in mononuclear cells activation in cases of bacteremia caused by these microorganisms.

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