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Effect of Xanthine Derivates and Dexamethasone on *Streptococcus pneumoniae*-Stimulated Production of Tumor Necrosis Factor Alpha, Interleukin-1β (IL-1β), and IL-10 by Human Leukocytes

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The present study concerns the release of the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha and of the anti-inflammatory cytokine IL-10 by human leukocytes in whole blood during stimulation with *Streptococcus pneumoniae* and the effects of various xanthine derivates, i.e., pentoxifylline (PTX), caffeine, and theofylline, and of dexamethasone (DXM). All three xanthine derivates and DXM inhibited the release of tumor necrosis factor alpha, PTX being the most effective. PTX, theofylline, and DXM inhibited the release of IL-1β, but caffeine did not affect IL-1β release. The release of IL-10 was significantly reduced by PTX at 24 h and by caffeine at 48 h, but DXM increased the release of this cytokine. In sum, the results of this study demonstrate that DXM inhibits only the release of proinflammatory cytokines but not of the anti-inflammatory cytokine IL-10 by human leukocytes, while PTX is the most potent inhibitor of both proinflammatory and anti-inflammatory cytokines.

Streptococcus pneumoniae is a gram-positive bacterium which can cause a variety of serious diseases such as otitis media, pneumonia, and meningitis (7). Knowledge about the production of cytokines during an infection with pneumococci is rather limited. Experimental pneumococcal meningitis in rabbits is the best-studied model (24, 31–34). The polysaccharide capsule of *S. pneumoniae* contributes to the invasiveness of this microorganism (30), while cell wall components, e.g., peptidoglycan and teichoic acid, play an important role in inducing meningeal inflammation (31–34) through stimulation of the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) (24). In experimental meningitis, the permeability of the blood-brain barrier is increased by TNF-α but not by IL-1β, which leads to an inflammatory exudate and cerebral edema (24). In the cerebrospinal fluid of neonates and children with pneumococcal meningitis, both TNF-α and IL-1β occur, and high levels of these cytokines in cerebrospinal fluid correlate with the development of neurological sequelae (2, 20). Besides proinflammatory cytokines, the anti-inflammatory cytokine IL-10 has also been detected in the cerebrospinal fluid of children with bacterial meningitis (11, 36). This cytokine inhibits the production of TNF-α (9, 12, 36), while TNF-α enhances the production of IL-10 by monocytes, providing a negative feedback mechanism (35, 39).

During treatment with antibiotics of bacterial meningitis caused by gram-positive or gram-negative bacteria, bacterial surface components are released into the subarachnoidal space. These compounds enhance the inflammatory response and stimulate the production of proinflammatory cytokines (1, 14, 22, 32, 40). Although antibiotics are of primary importance in the treatment of bacterial meningitis, experimental and clinical studies have focused on adjunctive therapy which might improve the clinical outcome. The compounds involved in these studies are pentoxifylline (PTX) and glucocorticosteroids, which have an inhibitory effect on the release of proinflammatory cytokines (16–18, 21, 23, 37, 41). The effects of these compounds on the release of IL-10 have not yet been studied. In a previous study, in which we stimulated human leukocytes with the gram-negative bacterium *Haemophilus influenzae*, the release of proinflammatory mediators could be inhibited by PTX and dexamethasone (DXM) (37). However, little about the effects of various xanthine derivates and DXM on the release of proinflammatory and anti-inflammatory cytokines by leukocytes during stimulation with gram-positive bacteria is known. The aim of the present study was to compare the effects of the xanthine derivates, PTX, theofylline, and caffeine, and of DXM on the release of TNF-α, IL-1β, and IL-10 by human leukocytes during stimulation with *S. pneumoniae* in vitro.

MATERIALS AND METHODS

Microorganisms. *S. pneumoniae* (serotype 6) was cultured at 37°C in brain heart infusion for 18 h. The bacteria were collected by centrifugation (10 min; 1,500 × *g*), washed twice with pyrogen-free saline, killed by incubation at 70°C for 1 h, and suspended at appropriate concentrations in saline.

Stimulation of cytokine release. Fresh heparinized (10 U/ml) blood from healthy volunteers was diluted fivefold with RPMI 1640 (Gibco BRL, Paisley, Scotland) containing 10^2 U of penicillin per ml, 50 μg of streptomycin per ml, 300 μg of L-glutamine per ml, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (hereafter called medium). Aliquots (1 ml) of blood were incubated with 10^6 heat-killed *S. pneumoniae* cells in 24-well tissue culture plates (Costar, Cambridge, Mass.) at 7.5% CO_2 at 37°C for 6, 24, and 48 h. Thereafter, the suspension was centrifuged (10 min; 1,500 × *g*), and the resulting supernatant was collected, filtered (Millipore Corp., Bedford, Mass.), and used to quantify the cytokines under study.

Drugs. PTX (Hoechst AG, Wiesbaden, Germany), caffeine (Pharmacie University Hospital, Leiden, The Netherlands), theofylline (Sigma Chemical Co., St. Louis, Mo.), and DXM (MSD, Haarlem, The Netherlands) were diluted in pyrogen-free saline and used in various concentrations as indicated in Results.

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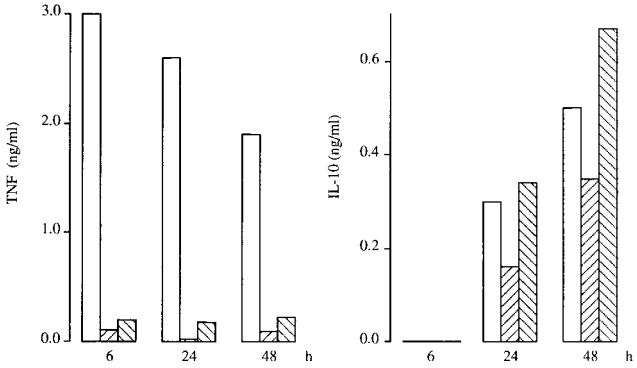

FIG. 1. Course of the release of TNF-α and IL-10 by leukocytes in whole blood stimulated with 10^6 heat-killed *S. pneumoniae* cells in the absence of PTX (□) or in the presence of 200 μg of PTX per ml (▨) or 1 μg of DXM per ml (▧). The values represent the mean cytokine levels of six experiments.

Measurements of cytokines. The concentration of IL-1β in plasma was determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Bucks, United Kingdom). The concentration of TNF-α in plasma was measured by ELISA (BPRC-TNO, Rijswijk, The Netherlands). In short, a microtiter plate (Titertek; Flow Laboratories, Zwanenburg, The Netherlands) was coated with 0.5 μg of purified capture anti-human TNF-α monoclonal antibody (BPRC-TNO) per well and incubated overnight at 4°C. After the wells were washed with Tris-buffered saline containing 0.05% Tween 20, 250 μl of phosphate-buffered saline (pH 7.4) containing 2% bovine serum albumin was added to the wells and incubated for 1 h at 37°C. Recombinant human TNF-α (Genzyme, Cambridge, Mass.) was used as a standard. One hundred microliters of supernatant or standard was added to the wells and incubated for 2 h at 37°C, and after the wells were washed, 0.15 μg of biotinylated anti-human TNF-α monoclonal antibody (BPRC-TNO) was added to each well and incubated for 1 h at 37°C. After being washed, the wells were incubated with 25 mU of anti-biotin-alkaline phosphatase (Boehringer, Mannheim, Germany) per well for 1 h at 37°C and washed, and 50 μg of *p*-nitrophenylphosphate disodium (Sigma) in substrate buffer (0.1 M glycine-NaOH, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4) was added to each well and incubated for 30 min at 37°C. The enzymatic reaction was stopped after 30 min by adding 30 μl of 3 M NaOH per well, and the A_{405} was read.

The concentration of IL-10 in plasma was measured by ELISA (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions, using a capture anti-human IL-10 monoclonal antibody (JES3-9D7) in a concentration of 0.1 μg per well and a biotinylated anti-IL-10 antibody (JES3-12G8) in a concentration of 0.1 μg per well. Tetramethylbenzidine was used as the substrate, and after termination of the reaction, the A_{450} was read.

Statistical analysis. Since the amount of cytokines released in whole blood from different donors varied, the effect of various drugs is expressed as the percentage of inhibition (mean and standard deviation) of three to nine experiments.

The percentage of inhibition is calculated according to the following equation: $(A - B)/(A - C) \times 100$ in which A is the cytokine level during stimulation with bacteria, B is the cytokine level during stimulation with bacteria in the presence of a drug, and C is the cytokine level in whole blood without stimulation and a drug. Values of more than 100% indicate that the amount of cytokine released by leukocytes during stimulation with bacteria in the presence of a drug (B) is smaller than the amount of cytokine released without stimulation and a drug (C). Negative values indicate that the amount of cytokine released during bacterial stimulation in the presence of a drug (B) is larger than that obtained during bacterial stimulation in the absence of a drug (A). The difference between the effects of various kinds of treatment on cytokine production was assessed by nonparametric Friedman analysis. The level of significance was set at 0.05.

RESULTS

Release of various cytokines by leukocytes in whole blood stimulated by *S. pneumoniae*. Incubation of whole blood with *S. pneumoniae* resulted in a time-dependent release of various proinflammatory and anti-inflammatory cytokines into the culture supernatant. The concentration of TNF-α was high (3.0 ± 1.1 ng/ml) at 6 h and decreased gradually thereafter, being 2.6 ± 1.8 ng/ml at 24 h and 1.9 ± 0.7 ng/ml at 48 h (Fig. 1). After 6 h of incubation, IL-1β was present (0.6 ± 0.3 ng/ml) in the supernatant, with a peak level at 24 h (3.3 ± 1.4 ng/ml), after which time the concentration declined to 1.9 ± 0.9 ng/ml at 48 h. IL-10 could not be detected at 6 h, was present at 24 h (0.3 ± 0.1 ng/ml), and increased further to 0.5 ± 0.3 ng/ml at 48 h (Fig. 1). On the basis of these results, in subsequent experiments the concentration of IL-10 was determined only at 24 and 48 h, while concentrations of TNF-α and IL-1β were also determined at 6 h.

Effect of PTX on the release of cytokines. The effect of PTX on the release of TNF-α during incubation of whole blood with *S. pneumoniae* was dose dependent, i.e., at 6 h the percentage of inhibition with 200 μg of PTX per ml was 112 ± 10 ($P < 0.05$), with 100 μg of PTX per ml it was 90 ± 7 ($P < 0.05$), and with 20 μg of PTX per ml it was 59 ± 7 ($P < 0.05$). The suppressive effect on TNF-α production remained high during 24 and 48 h of incubation, with percentages of inhibition of more than 100 (Fig. 1; Table 1). Two hundred micrograms of PTX per ml significantly reduced the release of IL-1β at 6, 24, and 48 h (Table 1); lower concentrations of PTX did not affect the release of IL-1β. The release of IL-10 at 24 h was also significantly inhibited by 200 μg of PTX per ml, although the percentage of inhibition was not as high as for TNF-α and IL-1β (Fig. 1; Table 1). At 48 h, the release of IL-10 was slightly, but not significantly, inhibited by PTX.

Effect of theofylline on the release of cytokines. Whole blood incubated with *S. pneumoniae* and 100 or 200 μg of theofylline per ml exhibited a significant decrease in the release of TNF-α after 6 h of incubation (Table 1). After 24 and 48 h, the inhibitory effect of 200 μg of theofylline per ml on the release of TNF-α had further increased significantly, with percentages of inhibition of more than 100 (Table 1). Theofylline in a concentration of 200 μg/ml also had a significant ($P < 0.05$) inhibitory effect on the release of IL-1β at 24 and 48 h but not at 6 h, at which time theofylline caused a slight but not significant increase in the IL-1β level. The release of IL-10 was not affected by theofylline at all time points studied (Table 1).

Effect of caffeine on the release of cytokines. A concentration of 200 μg of caffeine per ml reduced the release of TNF-α significantly at 6, 24, and 48 h (Table 1). Caffeine had no effect on the release of IL-1β at the various time points (Table 1),

TABLE 1. Percentage of inhibition of the release of TNF-α, IL-1β, and IL-10 in whole blood stimulated with *S. pneumoniae* by xanthine derivates or DXM[a]

Cytokine	Time (h)	No. of expts	% of inhibition with[b]:			
			PTX	THEO	CAF	DXM
TNF-α	6	3	110 (10)[c]	100 (20)[c]	80 (10)[c]	110 (10)[c]
	24	8	130 (30)[c]	130 (30)[c]	110 (20)[c]	120 (20)[c]
	48	9	110 (20)[c]	110 (20)[c]	110 (20)[c]	110 (30)[c]
IL-1β	6	6	60 (1)[c]	−20 (30)	−30 (20)	120 (20)[c]
	24	4	90 (1)[c]	80 (2)[c]	2 (10)	90 (10)[c]
	48	4	70 (1)[c]	40 (10)[c]	30 (30)	100 (30)[c]
IL-10[d]	24	5	80 (20)[c]	20 (60)	40 (50)	−10 (30)
	48	9	40 (50)	30 (50)	60 (40)[c]	−60 (90)

[a] Results are expressed as the percentage of inhibition according to the formula $(A - B)/(A - C) \times 100$ (see Materials and Methods), with the standard deviation in parentheses. When values are larger than 100%, B is smaller than C; when values are negative, B is larger than A. Statistical analysis with the original data on cytokine levels was performed by nonparametric Friedman analysis.

[b] The concentrations of the drugs used are as follows: 200 μg/ml for PTX, theofylline (THEO), and caffeine (CAF); 1 μg/ml for DXM.

[c] Cytokine levels significantly less ($P < 0.05$) than in whole blood stimulated with bacteria only.

[d] Not detectable at 6 h.

while the level of IL-10 was moderately, but significantly ($P < 0.05$), reduced by 200 μg of caffeine per ml at 48 but not at 24 h (Table 1).

Effect of DXM on the release of cytokines. DXM caused a significant dose-dependent decrease in the release of TNF-α after 6 h of incubation, with an optimal suppression when 1 μg of DXM per ml was used (with a percentage of inhibition of more than 100) (Fig. 1; Table 1). The suppressive effect of 1 μg of DXM per ml on TNF-α production remained high at 24 and 48 h (Fig. 1; Table 1). At 6, 24, and 48 h of incubation, DXM significantly reduced the release of IL-1β (Table 1). Incubation with 1 μg of DXM per ml did not inhibit the release of IL-10 in whole blood stimulated with *S. pneumoniae* at 24 and 48 h. In contrast, we found an increase in the amount of IL-10 released after DXM treatment (Fig. 1), resulting in negative percentages of inhibition (Table 1).

Comparison of the efficacies of the different drugs to inhibit cytokine production. The efficacies of the various drugs to inhibit the release of cytokines at 6, 24, and 48 h were compared. PTX was the drug most effective ($P < 0.005$) in inhibiting the release of TNF-α by *S. pneumoniae*-stimulated leukocytes. PTX and DXM were equally effective in inhibiting IL-1β release. The release of IL-10 was inhibited only by PTX and caffeine, and there was no difference in the efficacies of these two drugs.

DISCUSSION

The main conclusion to be drawn from the present study is that DXM significantly reduced the release of the proinflammatory cytokines TNF-α and IL-1β and increased the release of the anti-inflammatory cytokine IL-10 by leukocytes stimulated with heat-killed *S. pneumoniae*, whereas PTX significantly reduced the release of both proinflammatory and anti-inflammatory cytokines. The effects of theofylline and caffeine on cytokine release were variable.

In the whole-blood assay, TNF-α and IL-1β are formed mainly by monocytes (37); IL-10 is formed by monocytes and T lymphocytes (19). Since these cells migrate from the blood to a site of inflammation, the present findings are relevant for the additive therapy of bacterial meningitis. The development of neurological sequelae in patients with bacterial meningitis may occur via a toxic effect of TNF-α on neuronal cells due to high cytokine levels in the cerebrospinal fluid of these patients (26, 29). Thus, inhibition of TNF-α can be beneficial in preventing the occurrence of sequelae. Since TNF-α upregulates adhesion molecules on endothelial cells such as ICAM-1, inhibition of TNF-α release will reduce the influx of monocytes and neutrophils to the site of inflammation as well (4, 38). In our study we used, besides PTX, the xanthine derivates caffeine and theofylline, to compare their effects. The results demonstrate an inhibitory effect of all three xanthine derivates on the production of TNF-α by human leukocytes stimulated with *S. pneumoniae*. This is in agreement with earlier studies demonstrating that PTX has an inhibitory effect on the release of TNF-α by monocytes after stimulation with gram-negative bacteria or lipopolysaccharide (10, 13, 23, 25, 27, 37). The exact mechanism by which xanthine derivates suppress the release of TNF-α is unknown. They might act via their nonspecific inhibitory effect on the activity of intracellular phosphodiesterases, which increase the level of cyclic AMP (5, 10, 27, 28), and PTX might inhibit the transcription of TNF-α mRNA (13, 25, 28). The present results show that xanthine derivates are not specific inhibitors of TNF-α, because PTX and theofylline also decreased the release of IL-1β by leukocytes, which has also been found for murine microglial cells (8). Moreover, the present study demonstrates that PTX and caffeine inhibit IL-10 production by stimulated leukocytes.

We also investigated the influence of DXM on proinflammatory and anti-inflammatory cytokine release by *S. pneumoniae*-stimulated leukocytes. Despite the inhibitory effect of DXM on the production and release of proinflammatory cytokines, the amount of IL-10 released was increased. An increase in the release of any cytokine by DXM has not been reported earlier. The inhibition of proinflammatory cytokines and stimulation of an anti-inflammatory cytokine indicates another anti-inflammatory mechanism of glucocorticosteroids. The exact mechanism of action of DXM on cytokine production is not yet certain; downregulation of the proinflammatory mediators by DXM might be caused by inhibition of transcription factors (3) or by inhibition of the translation of mRNA (13). The mechanism underlying the stimulation of IL-10 release remains to be elucidated.

Comparison of the effects of PTX and DXM on the production of proinflammatory and anti-inflammatory cytokines by stimulated leukocytes could offer insight into possible differences in intracellular signal transduction pathways. In a previous study in which whole blood was stimulated with *S. pneumoniae* or *H. influenzae* in the presence of neutralizing IL-10 monoclonal antibody, we demonstrated that endogenous IL-10 limits the production of TNF-α by human leukocytes (36), as has been demonstrated after stimulation by lipopolysaccharide (6, 12, 15). In order to reduce the release of TNF-α in vivo during an infection, it could be advisable to use glucocorticosteroids, since these drugs do not reduce the release of IL-10, which possibly keeps in check the production and release of TNF-α. Since PTX also has an inhibitory effect on the release of IL-10, it might be less favorable for use during an infection.

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