The effects of dexamethasone and chlorpromazine on tumour necrosis factor-\(\alpha\), interleukin-1\(\beta\), interleukin-1 receptor antagonist and interleukin-10 in human volunteers

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SUMMARY
Tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1\(\beta\) (IL-1\(\beta\)) are pro-inflammatory cytokines that play an important role in severe infections, whereas IL-1 receptor antagonist (IL-1ra) and IL-10 are anti-inflammatory cytokines that counteract their effects. Chlorpromazine and dexamethasone protect mice against lethal endotoxaemia by decreasing circulating concentrations of TNF-\(\alpha\) and IL-1\(\beta\). We investigated whether administration of chlorpromazine or dexamethasone to human volunteers is able to modulate the lipopolysaccharide (LPS)-stimulated cytokine production capacity in whole blood. Blood samples were taken before and several time-points after medication. Circulating cytokine concentrations were low in all samples. LPS-induced TNF-\(\alpha\) and IL-1\(\beta\) production in whole blood was inhibited by dexamethasone treatment, while chlorpromazine had no effect. When peripheral blood mononuclear cells were stimulated \textit{in vitro} with LPS, the addition of chlorpromazine (1-100 ng/ml) had no modulatory action on TNF-\(\alpha\), IL-1\(\beta\), IL-1ra or IL-10 synthesis. The chlorpromazine concentrations measured in circulation of volunteers were eight to 40 times lower than the concentrations shown to be effective in mice. In conclusion, chlorpromazine inhibits TNF-\(\alpha\) and IL-1\(\beta\) production in mice at concentrations that cannot be reached in humans, thus precluding its usage in clinical anti-cytokine strategies. In contrast, dexamethasone is an effective inhibitor of pro-inflammatory cytokine production.

INTRODUCTION
The pro-inflammatory cytokines interleukin-1\(\beta\) (IL-1\(\beta\)) and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) play an important role in the pathogenesis of sepsis.\(^1\) Blocking TNF improves survival in mice receiving a lethal endotoxin challenge\(^2\) and in baboons challenged with an injection of \textit{Escherichia coli} bacteria,\(^3\) while administration of IL-1 receptor antagonist (IL-1ra), a naturally occurring antagonist of IL-1, has been shown to prevent experimental septic shock.\(^4\) Therefore, pharmacological agents that inhibit TNF-\(\alpha\) and IL-1\(\beta\) synthesis may prove beneficial in the treatment of severe infections.

Glucocorticoids are important anti-inflammatory agents. This effect is partly due to attenuation of the production of pro-inflammatory cytokines. Dexamethasone suppresses cytokine production by lipopolysaccharide (LPS)-stimulated human monocytes,\(^5,6\) and adrenalectomy sensitizes mice to the lethal effects of IL-1 and TNF.\(^7\) In lethal endotoxaemia, administration of dexamethasone was able to inhibit cytokine production and to increase survival.\(^5,9\) In healthy volunteers, glucocorticoids attenuated TNF production and the haemodynamic response when given immediately before LPS.\(^10\)

Chlorpromazine, a phenothiazine derivative, is also able to protect mice against the effects of endotoxin through attenuation of serum levels of cytokines.\(^8,9,11\) \textit{In vitro}, chlorpromazine is able to inhibit TNF-\(\alpha\) synthesis by human monocytes.\(^12\) In addition, recent studies reported stimulatory effects of chlorpromazine on the production of IL-10, an anti-inflammatory cytokine able to inhibit the production of TNF-\(\alpha\) and IL-1\(\beta\).\(^13,14\) Thus, chlorpromazine could be a useful drug for cytokine modulation in humans.

The aim of this study was to investigate the capacity of chlorpromazine to modulate cytokine synthesis in humans, by administration of chlorpromazine to healthy volunteers and measurement of the \textit{ex vivo} LPS-stimulated production of TNF-\(\alpha\), IL-1\(\beta\), IL-1ra and IL-10. The effect of chlorpromazine was compared to that of dexamethasone.

MATERIALS AND METHODS
Ex vivo studies in humans
Eighteen healthy volunteers (five females and 13 males, mean age 24 years, range 19–36 years) participated in the study, after giving written informed consent. The study was approved by the ethics committee of the University Hospital Nijmegen.
The volunteers refrained from heavy exercise, since this was shown to modulate cytokine production, and did not use any medication. They were randomized to receive chlorpromazine (25 mg t.i.d.; Brocacef, Maarsen, the Netherlands), dexamethasone [1.5 mg three times a day (t.i.d.); Genpharma, Maarsen, the Netherlands], or placebo at 8-h intervals. Blood samples were drawn 5 hr before treatment and 1, 4 and 25 hr after taking the third dose. All samples were drawn at 9 a.m. while subjects fasted, except the third sample which was obtained at noon after a small standard meal.

Ex vivo whole blood production assay was performed as described elsewhere, with minor modifications. Briefly, blood was collected in one 4-ml and two 2-ml EDTA tubes (Vacutainer systems, Becton Dickinson, Rutherford, NJ). The first tube was processed immediately and used for measuring circulating levels of TNF-α, IL-1β, IL-1ra and IL-10. In the other tubes, ex vivo cytokine production was determined; one tube was incubated at 37° with lipopolysaccharide (LPS; 10 μg/ml; Escherichia coli 055:B5; Sigma Chemical Co., St Louis, MO) for 24 hr and the other without additional stimuli. After incubation, the tubes were centrifuged at 10 000 g for 10 min. The plasma was separated and centrifuged at 15 000 g for 5 min to obtain platelet-poor plasma. Blood for chlorpromazine measurement was drawn into 10-ml tubes (Venoject II, Terumo Europe, Leuven, Belgium), and plasma obtained by centrifugation (10 min, 10 000 g). Aliquots were stored at −20°C until assay.

Three of the volunteers receiving chlorpromazine took part in an additional study to assess whether chlorpromazine administration in vivo can modify cytokine production by isolated peripheral blood mononuclear cells (PBMC). Chlorpromazine was administered as described above and blood was collected 5 hr before the treatment was started and 1 hr after the third dose. Isolation of PBMC was performed as described elsewhere, with minor modifications. Briefly, venous blood was drawn into 10-ml EDTA tubes (Monoject, 's-Hertogenbosch, the Netherlands). The PBMC fraction was obtained by density centrifugation of blood (diluted 1:1 in pyrogen-free saline) over Ficoll–Paque (Pharmacia Biotech AB, Uppsala, Sweden). PBMC were washed twice in saline, and suspended in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals Inc., Costa Mesa) supplemented with human serum 5%, gentamicine 1%, t-glutamine 1%, pyruvate 1%. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, the Netherlands) and the number was adjusted to 5 × 10⁶ cells/ml. One hundred microlitres were incubated in 96-well plates (Greiner B.V., Alphen a/d Rijn, the Netherlands) with LPS (final concentration 1 ng/ml) for 24 hr at 37°C. After 24 hr of incubation the supernatants were frozen (−20°C) until assay.

In vitro effect of chlorpromazine
In order to assess the effect of chlorpromazine without interference of in vivo pharmacokinetics, the in vitro effect of chlorpromazine on cytokine production was investigated. Whole blood or PBMC were obtained from seven volunteers as described above. All volunteers were healthy and received no treatment. LPS stimulation of PBMC or whole blood cultures was performed for 24 hr at 37°C, as described above, in the presence or absence of chlorpromazine (1–10 ng/ml). Additionally, the effect of chlorpromazine at a concentration of 100 ng/ml on cytokine production was tested in LPS-stimulated PBMC from three volunteers. Samples were stored at −20°C until assay.

Studies in mice
Mice (C57B1/6J mice, 8 weeks old, weight 20–25 g) were given chlorpromazine (4 mg/kg) in pyrogen-free saline by intraperitoneal (i.p.) injection, 30 min before challenge with LPS (30 μg/kg intraperitoneally). This dose has been shown to be optimal for the chlorpromazine-induced protection in lethal endotoxaemia. After 90 min, the mice were anaesthetized with ether and bled from the retroorbital plexus. Blood was pooled into two 10-ml tubes (Venoject) and serum was stored at −20°C until measurement of the plasma concentration of chlorpromazine.

Cytokine measurements
TNF-α, IL-1β and IL-1ra concentration were determined by radioimmunoassay as described elsewhere. IL-10 concentrations were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (CLB, Amsterdam, the Netherlands), according to the instructions of the manufacturer. In order to correct for spontaneous cytokine release, the net production was calculated by subtracting the values in the unstimulated samples from the concentration obtained after LPS stimulation.

Chlorpromazine measurement
Concentrations of chlorpromazine were measured at the Department of Pharmacy, St Elisabeth Hospital Venray, the Netherlands, with one-step extraction followed by high-pressure liquid chromatography. In short, 1 ml of serum was mixed with 0.4 ml of carbonic buffer (pH 9.4), 2.0 ml heptane:iso-amyl alcohol (985:15), and 50 μl of Northiaden 50 μg/l. After centrifugation, the heptane phase was transferred and dried at 60°C. Chromatography was performed on a Spherisorb SSW with guard column. The mobile phase consisted of MeOH/Acetonitril/25% ammonia (20/280/1), the flow was 1.4 ml/min and ultra-violet light was used for detection.

Statistical analysis
Data are shown as mean ± SEM. Differences between curves were tested using ANOVA for repeated measures. Differences at time-points between more than two groups were tested with ANOVA. Differences in production of cytokines between drug and placebo were analysed using non-parametric Wilcoxon test. Results were considered statistically significant at P < 0.05.

RESULTS
Effect of orally applied chlorpromazine and dexamethasone on cytokine production
Five of the volunteers reported side-effects of the medication. One of the volunteers receiving placebo felt dizzy. Four subjects taking chlorpromazine experienced drowsiness and one reported abdominal pain. Circulating concentrations of TNF-α, IL-1β, IL-1ra and IL-10 were very low and in the normal range in all samples, and were not altered by the treatment with either of the two drugs (range TNF-α < 40–100 pg/ml; IL-1/β < 20–90 pg/ml; IL-1ra 80–400 pg/ml; IL-10 < 38 pg/ml).
Chlorpromazine treatment did not influence either the TNF-α or the IL-1β production capacity in whole blood at any of the time-points tested (Fig. 1a, b). Neither of the drugs had any effect on IL-1ra synthesis (Fig. 1c). Dexamethasone significantly inhibited \textit{ex vivo} whole blood production of TNF-α both 1 and 4 hr ($P<0.01$), but not 24 hr, after last administration (Fig. 1a). Synthesis of IL-1β was inhibited 1 hr ($P<0.05$), but not 4 and 24 hr, after the last dexamethasone dose (Fig. 1b). IL-10 concentrations with and without LPS-stimulation were very low and not stimulated by treatment with chlorpromazine or dexamethasone (data not shown).

Since chlorpromazine had no effect on cytokine production in whole blood cultures, we investigated whether this could be due to interference by the various cell types present in circulation, or other plasma factors. The LPS-stimulated cytokine production of PBMC isolated from volunteers 1 hr after the last dose of chlorpromazine did not differ from their cytokine production capacity 5 hr before the start of the treatment (data not shown), suggesting that interference of plasma factors with chlorpromazine in the whole blood assay was not responsible for the lack of effect of the drug.

\textbf{Circulating concentrations of chlorpromazine}

The serum concentrations of chlorpromazine in human volunteers were 2.5±0.3 ng/ml at 1 hr, 1.5±0.4 at 3 hr and 1.2±0.3 at 25 hr after the last administration of chlorpromazine. In mice, i.p. treatment with 4 mg/kg chlorpromazine, which was shown to inhibit cytokine production by us and by others,\textsuperscript{6,11} resulted in circulating chlorpromazine concentrations of 41.0±3.0 ng/ml at 90 min after infection, i.e. 8–40 times higher than the concentrations in human volunteers.

\textbf{In vitro effects of chlorpromazine}

The effect of \textit{in vitro} addition of chlorpromazine to LPS-stimulated PBMC and whole blood cultures was studied. Chlorpromazine concentrations that can be reached by oral administration of the drug in humans (1 and 10 ng/ml) were used. At these concentrations, there was no effect on cytokine production by LPS-stimulated PBMC (Fig. 2) or whole blood cultures (data not shown). Moreover, in an additional experiment, the chlorpromazine concentration of 100 ng/ml (20-times higher than the maximum chlorpromazine concentration measured in volunteers) was also not effective for modulation of LPS-stimulated cytokine production by PBMC, when compared with non-treated cells (TNF-α, 0.9±0.5 versus 1.1±0.7 ng/ml; IL-1β, 2.4±0.7 versus 2.3±0.5 ng/ml; IL-1ra, 9.7±1.6 versus 12.9±4.4 ng/ml; IL-10, 0.3±0.15 versus 0.4±0.1 ng/ml; $P>0.05$ for all cytokines).

\textbf{DISCUSSION}

The results of the present study show that chlorpromazine administered \textit{in vivo} to human volunteers does not inhibit the production of TNF-α, IL-1β and IL-1ra, and has no stimulatory effect on IL-10 synthesis. In contrast, dexamethasone has a potent inhibitory action on TNF-α and IL-1β production capacity.

The inhibiting effect of dexamethasone on pro-inflammatory cytokine production has been shown both \textit{in vitro}\textsuperscript{5,6} and in human volunteers.\textsuperscript{10} The mechanism by which dexamethasone inhibits cytokine synthesis involves transcriptional activation of IκBα, which binds and inactivates the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Dexamethasone (DEX), but not chlorpromazine (CPZ) treatment, significantly inhibited production of TNF-α (a) and IL-1β (b) by LPS-stimulated whole blood cultures of human volunteers. Neither dexamethasone nor chlorpromazine treatment had any effect on IL-1ra production (c).}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Chlorpromazine (CPZ) had no effect on LPS-stimulated cytokine production capacity by isolated human PBMC \textit{in vitro}.}
\end{figure}
transcription factor NF-kB, blocking its translocation to the nucleus and thus markedly reducing cytokine synthesis. Our results show a marked inhibition of TNF-α and IL-1β synthesis in LPS-stimulated whole blood cultures of volunteers treated with dexamethasone. TNF-α production was diminished at both 1 and 4 hr after the last administered dose. IL-1β production was significantly inhibited only after 1 hr, although a tendency of a decreased IL-1β production was also seen 4 hr after the last administration of dexamethasone. In contrast, IL-1ra production was not affected by dexamethasone treatment. These data are corroborated by the results obtained by Santos et al. in an in vivo study in human volunteers. In contrast, recent studies have shown an inhibitory effect of hypercortisolaemia on IL-1ra response after LPS challenge. Recent studies have shown an inhibitory effect of chlorpromazine on IL-1 production by human mononuclear cells. In contrast, several experimental studies in animals have shown that chlorpromazine protected animals from lethal endotoxaemia by inhibition of LPS-induced pro-inflammatory cytokine response and stimulation of the anti-inflammatory cytokine IL-10. A major difference between our study and the above-mentioned experimental studies in animals have shown that chlorpromazine did not influence LPS-stimulated cytokine synthesis in whole blood. No effect on cytokine production by PBMC was observed either, arguing against interference of chlorpromazine with plasma factors in syntheses by PBMC. The results obtained by Martinez et al. have also failed to demonstrate an inhibitory effect of chlorpromazine on IL-1 production by human mononuclear cells. In contrast, several experimental studies in animals have shown that chlorpromazine protected animals from lethal endotoxaemia by inhibition of LPS-induced pro-inflammatory cytokine response and stimulation of the anti-inflammatory cytokine IL-10. A major difference between our study and the above-mentioned reports is the chlorpromazine concentrations in the circulation of the human volunteers, which was eight to 40 times lower than the concentrations observed in mice. In vitro studies performed on human monocytes have shown inhibitory effects of chlorpromazine on cytokine mRNA expression or production. However, the chlorpromazine concentrations used in these studies were more than 100 times higher than the chlorpromazine circulating concentrations we measured in the volunteers receiving the drug. The lack of effect of chlorpromazine in our study cannot be completely attributed to differences in plasma concentrations between mice and humans, because the concentrations used by Martinez et al. and the 100 ng/ml chlorpromazine concentration used in vitro in some of our experiments were higher than the concentrations recorded in mice, while still having no effect on cytokine production by human PBMC. In this respect, it should be mentioned that as many as 10 or 12 metabolites of chlorpromazine occur in circulation in appreciable quantities, many being active. Differences in the kinetics of chlorpromazine and in the drug metabolites in mice and humans may be also responsible for some of the differences observed. In conclusion, in contrast with dexamethasone, which has a potent inhibitory effect on cytokine synthesis, administration of chlorpromazine did not influence LPS-stimulated cytokine production capacity in whole blood or PBMC of human volunteers, thus precluding its direct usage in clinical strategies aimed to inhibit TNF-α and IL-1β.

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REFERENCES


