Chlorpromazine specifically inhibits peripheral and brain TNF production, and up-regulates IL-10 production, in mice

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SUMMARY

We have previously shown that chlorpromazine (CPZ) inhibits tumour necrosis factor (TNF) production and protects against endotoxic shock in mice. In this paper we investigated the effect of pretreatment with CPZ, 4 mg/kg i.p. 30 min before, compared with dexamethasone (DEX; 3 mg/kg) on the induction of other endotoxin (lipopolysaccharide; LPS)-induced cytokines in the serum of mice, i.e. interleukin-1α (IL-1α), IL-6 and IL-10, and TNF. We also studied the effect of CPZ on serum and spleen-associated TNF. Both DEX and CPZ inhibited TNF production, whereas induction of IL-1 and IL-6 was inhibited by DEX but not by CPZ. DEX did not affect IL-10, while CPZ potentiated its induction. CPZ also inhibited spleen-associated TNF induction in LPS-treated mice, suggesting an effect on the synthesis of TNF. CPZ inhibited TNF induction by Gram-positive bacteria (heat-killed *Staphylococcus epidermidis*) and by anti-CD3 monoclonal antibodies. Intraperitoneal administration of CPZ also inhibited the induction of brain-associated TNF induced by intra-cerebroventricular injection of LPS. Therefore, CPZ is a more specific inhibitor of TNF production than DEX; in particular, CPZ increased the induction of IL-10, which is a 'protective' cytokine known to inhibit LPS toxicity and TNF production. CPZ inhibited TNF production *in vivo*, irrespective of the TNF stimulus used to induce TNF. Finally, CPZ did not induce the 'rebound' effect of DEX that, when given 24 hr before LPS, potentiates TNF production, but it did inhibit TNF production after 24 hr.

INTRODUCTION

Chlorpromazine (CPZ), a well-known anti-psychotic agent, inhibits tumour necrosis factor (TNF) production and protects against endotoxin (lipopolysaccharide; LPS) toxicity *in vivo*, with a potency comparable to that of glucocorticoids.[1-3] It was therefore of interest to characterize the specificity of CPZ towards TNF synthesis, and the aim of this work was to investigate the effect of CPZ pretreatment on the induction of other pro-inflammatory cytokines (interleukins IL-1 and IL-6). Recently, it was pointed out that IL-10 can inhibit TNF production *in vivo*, and the role of IL-10 as a feedback inhibitor of TNF production is suggested by papers showing that anti-IL-10 antibodies potentiate LPS-induced TNF production, while administration of recombinant IL-10 protects against endotoxic shock.[4,5] For this reason, we also studied the effect of CPZ on LPS-induced IL-10 levels.

Another point was to define whether CPZ inhibited just TNF release and not its production. We thus tested the effect of CPZ pretreatment on the induction of tissue-associated TNF.

An important feature of CPZ, a psychotropic drug, is its ability to concentrate in the brain,[6] and TNF has been proposed as a pathogenetic mediator in some diseases of the central nervous system, including cerebral malaria, bacterial meningitis and experimental allergic encephalomyelitis.[7-9] Therefore, we studied the effect of CPZ on TNF production within the brain following intra-cerebroventricular injection of LPS, which might mimic an infectious process in the brain.

We also wondered whether the inhibitory effect of CPZ on TNF production was specific for LPS-induced TNF. In fact, while LPS shock is a widely used animal model, in septic patients a mixed Gram-negative and Gram-positive infection is often present. For this reason, we have studied the effect of CPZ on serum TNF induced by an injection of heat-killed *Staphylococcus epidermidis* (Staph). A previous report has indicated that administration of heat-killed Staph to rabbits induced serum TNF levels by a mechanism that was somewhat different to that of LPS.[10] In particular, while LPS-induced TNF was unaffected by IL-1 receptor antagonist (IL-1ra), Staph induction of TNF was IL-1ra-inhibitable, indicating that

Staph induces TNF at least in part via IL-1. Furthermore, LPS from Gram-negative bacteria induced TNF by interacting with specific membrane receptors, obviously different from those mediating interactions with Gram-positive bacterial products, and it is likely that the signalling mechanism leading to activation of TNF production will be different.

Finally, we investigated whether CPZ could also inhibit TNF induction in mice treated with anti-CD3 monoclonal antibodies, using the 145-2C11 hamster anti-mouse antibody, which acts as a polyclonal T-cell activator. 145-2C11 induces toxicity and cytokine release syndrome in mice that can be ameliorated with glucocorticoids or anti-TNF antibodies.[11–13] In this respect, this could be an animal model for the toxicity of OKT3, a monoclonal antibody to human CD3 which is used as an immunosuppressive agent in transplanted patients and induces a series of adverse effects attributable to release of this cytokine. It was therefore useful to evaluate the possible inhibition of 145-2C11-induced TNF production by CPZ, not only to study its effect on TNF produced in response to a non-LPS stimulus, but also to obtain a useful induction of the amelioration of OKT3 toxicity.

MATERIALS AND METHODS

Animals and treatments

Male CD1 mice (25 g body weight) from Charles River Italia (Calco, Como, Italy) were used. They were housed five per cage and fed *ad libitum*. LPS (phenol-extracted preparation from *Escherichia coli* O55: B5; Sigma, St Louis, MO) was given intraperitoneally (i.p.) at a dose of 25 μg/mouse, unless otherwise indicated.

In some experiments, 2·5 μg of LPS in 5 μl of saline was injected intra-cerebroventricularly (i.c.v.) via a 28-gauge needle into ether-anaesthetized mice.[14]

The hamster monoclonal antibody 145-2C11, directed against mouse CD3, was prepared as described elsewhere[11] and used at a dose of 50 μg/mouse.

Heat-inactivated Staph, prepared as previously described,[10] was a kind gift from Charles A. Dinarello (Boston, MA). The bacterial suspension was adjusted to 10^{10} organisms/ml and mice were treated i.p. with 0·2 ml of this suspension.

CPZ (Farmitalia Carlo Erba, Nerviano, Italy) was given at a dose of 4 mg/kg, and DEX (dexamethasone phosphate; Merck, Darmstadt, Germany) at a dose of 3 mg/kg, 30 min before LPS. All injections were i.p. in sterile, pyrogen-free saline, and were done between 9.00 a.m. and 11.00 a.m. Blood was obtained from the retro-orbital plexus under light ether

anaesthesia, and serum was prepared. Blood was collected at 1 hr for TNF, 1·5 hr for IL-10 and 2 hr for IL-6 determination. For IL-1 determinations, spleen homogenates were prepared 3 hr after the LPS injection. Briefly, spleens were removed and homogenized with an Ultra Turrax in 10 vol (w/v) of ice-cold saline. The homogenate was then centrifuged for 30 min at 105,000 g and supernatant was used for IL-1 assay. Preliminary experiments indicated that these were the optimal time-points for the determination of different cytokines in LPS-treated mice.

Procedures involving animals and their care conformed with the institutional guidelines, which are in compliance with national and international laws and policies.[15,16]

Cytokine determination

TNF was measured by the degree of cytotoxicity on L929 cells in the presence of 1 μg/ml of actinomycin D, as previously described,[17] using human recombinant TNF as standard; the sensitivity of the assay was 12 pg/ml. When indicated, TNF in spleen homogenates was measured using the same method.

Serum IL-6 was measured as hybridoma growth factor using 7TD1 cells (a kind gift from Dr van Snick), as previously described.[18] IL-6 activity is expressed as co-stimulatory U/ml, using rIL-6 as a standard. The following recombinant cytokines were used to test the specificity of the IL-6 assay, and none was found to have hybridoma growth factor activity on 7TD1 cells: IL-1α and IL-1β, IL-2, IL-3, IL-4, granulocyte–macrophage colony-stimulating factor (GM-CSF); M-CSF, lymphotoxin, TNF-α as well as phorbol myristate acetate (PMA). The sensitivity of the assay was 50 U/ml.

IL-1α was measured in spleen homogenates by a commercially available radioimmunoassay (RIA; Cytokine Sciences Inc., Boston, MA).

IL-10 was measured by an ELISA, as previously described.[13]

RESULTS

Effect of CPZ and DEX on TNF, IL-1, IL-6 and IL-10 induction by LPS

Table 1 shows the peak serum levels of different cytokines in LPS-treated mice and the effect of a pretreatment with 4 mg/kg of CPZ. Experiments were repeated at least twice and data from a representative one are reported. Since no circulating IL-1α was detected within the sensitivity of the RIA used, IL-1α was measured in the spleen of LPS-treated mice. It can be seen that, while completely blocking TNF induction, CPZ did not affect

Table 1. Effect of CPZ pretreatment on LPS-induced cytokines

	LPS alone	CPZ + LPS	DEX + LPS
TNF (ng/ml)	108 ± 67	1·0 ± 0·3*	< 1
Spleen TNF (ng/g)†	67 ± 23	9 ± 4*	< 1
Spleen IL-1α (ng/g)†	2·1 ± 0·7	2·1 ± 1·4	< 0·4
IL-6 (U/ml)	78,301 ± 17,934	82,134 ± 23,481	36,363 ± 13,343*
IL-10 (U/ml)	13·5 ± 0·89	42·4 ± 19·5*	23·8 ± 2·8*

†Measured in spleen homogenates and expressed as ng/g of tissue. Data are mean ± SE of five mice per group.
*P < 0·05 versus LPS alone by Student's t-test.
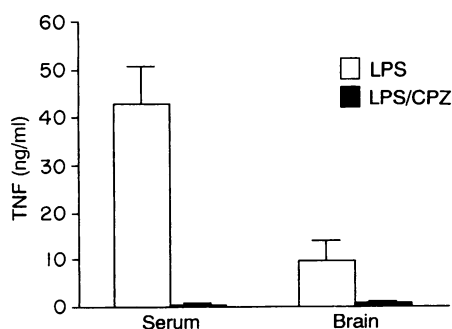
Figure 1. CPZ inhibits brain TNF production. Mice were treated with CPZ (4 mg/kg) 30 min before LPS (2·5 μg/mouse, i.c.v.). TNF was measured in serum and brain homogenates 1 hr after LPS. Data are mean ± SD (n = 5).

Table 2. Effect of CPZ and DEX on serum TNF induced by anti-CD3 or Staph

Treatment	Staph	Anti-CD3
Control	130 ± 79	53 ± 17
CPZ	5 ± 4*	25 ± 8*
DEX	<0·05*	6 ± 1*

Mice were treated with 50 μg of anti-CD3 or 0·2 ml of a suspension of heat-killed Staph (10^{10}/ml). Serum TNF was measured 1 hr later. Data are ng TNF/ml (mean ± SE; n = 5).
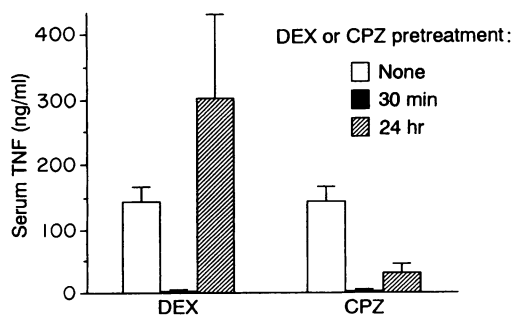*P < 0·05 versus control by Dunnet's test.

IL-6 or IL-1A, and markedly potentiated IL-10 induction. DEX, used as a reference inhibitor of TNF synthesis, inhibited TNF as well, but also IL-1α and, less markedly, IL-6, while it had no significant effect on IL-10 induction. No cytokines were detectable in sera or tissue from mice treated with saline or CPZ alone without LPS (data not shown). Table 1 also shows that CPZ inhibited the induction of spleen-associated TNF.

CPZ inhibition of brain TNF production

No TNF was detectable in brain homogenates from mice treated with LPS i.p. On the other hand, i.c.v. administration of LPS (2·5 μg/mouse) consistently induced the appearance of TNF in brain and in serum, with a peak 1 hr after injection (data not shown). As shown in Fig. 1, pretreatment with CPZ (4 mg/kg, 30 min before LPS) blocked this induction (P < 0·001 by Duncan's test).

Time-dependency of the effect of CPZ and DEX on TNF production

As shown in Fig. 2, while a 30-min pretreatment with DEX (10 mg/kg i.p.) inhibited serum TNF levels after i.p. injection of LPS, a 24-hr pretreatment had a potentiating effect on TNF production, as previously described.[19] In contrast, as shown in

the same figure, CPZ (4 mg/kg, i.p.) not only did not potentiate, but still inhibited TNF production.

CPZ and DEX inhibit TNF induction by heat-inactivated Staph

As shown in Table 2, CPZ and DEX also inhibited serum TNF levels induced by i.p. administration of 2 × 10^7 killed Staph or by anti-CD3 monoclonal antibodies.

DISCUSSION

TNF is considered to be a key mediator in the pathogenesis of endotoxic shock, as demonstrated by the protective effect of anti-TNF antibodies or soluble TNF receptors in various animal models.[2,20,21]

The present paper confirms the inhibitory effect of CPZ on TNF production, previously shown to result in protection against LPS lethality.[3] CPZ not only blocked the appearance of TNF in the serum, but also in spleen homogenates, indicating an effect on TNF production rather than its secretion.

The inhibitory action of CPZ on brain TNF production was expected, in view of its pharmacological propensity to accumulate in the brain. Centrally administered LPS induced TNF not only in the brain but also in the blood, as was described for IL-6.[22]

Compared with the other LPS-induced cytokines measured, CPZ showed a specificity for TNF: it did not inhibit IL-1α or IL-6 production. Furthermore, it inhibited serum TNF production even when this was induced with non-LPS stimuli. In particular, we found inhibition of anti-CD3 (145-2C11)-induced TNF, which might suggest the use of CPZ to prevent OKT3-associated toxicity, which is also TNF mediated.[11-13] CPZ also inhibited the induction of TNF by Gram-positive bacteria (heat-inactivated Staph), which is obviously acting via a different pathway to LPS. These data, together with the fact that not all the LPS-induced cytokines were inhibited by CPZ, support the idea that this drug is acting on a specific pathway of TNF induction and not just as a LPS antagonist.

CPZ was also found to potentiate LPS-induced IL-10 production, as was reported for cyclosporin A.[13] IL-10 has been shown to inhibit TNF release and protect mice against endotoxic shock, suggesting that endogenous IL-10 induced by LPS *in vivo* functions as an important protective mechanism against LPS toxicity. Therefore the lack of inhibition observed

Figure 2. Effect of a 30-min and a 24-hr pretreatment with DEX or CPZ on serum TNF levels. Mice were treated with DEX (10 mg/kg) or CPZ (4 mg/kg) 30 min or 24 hr before LPS (2·5 μg/mouse, i.p.). Serum TNF was determined 1 hr after LPS. Data are mean ± SD (n = 5).

with CPZ is certainly a desirable feature of a drug to be used in the therapy or prevention of TNF-mediated pathologies.

Twenty-four hours after treatment, CPZ still inhibited TNF production, while DEX pretreatment at 12 hr resulted in a potentiation of TNF production (probably due to desensitization of the hypothalamus–pituitary–adrenal axis).

In conclusion, the data reported here show that CPZ is a rather specific inhibitor of TNF production, irrespective of the agent used to induce TNF. It does not impair endogenous feedback systems, unlike DEX which induces a late potentiation of TNF production, and it up-regulates the induction of the protective cytokine IL-10.

ACKNOWLEDGMENTS

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