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Tetrandrine, a plant alkaloid, inhibits the production of tumour necrosis factor-alpha (cachectin) hy human monocytes

A. FERRANTE*, W. K. SEOW†, B. ROWAN-KELLY* & Y. H. THONG† *Department of Immunology and University Department of Paediatrics, Adelaide Children's Hospital, Adelaide, and †Immunobiology Laboratory, Department of Child Health, University of Queensland, Mater Children's Hospital, South Brisbane, Australia*

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SUMMARY

Human mononuclear leucocytes (MNL) or the adherent fraction (monocytes) produced tumour necrosis factor-alpha (TNF-α) (by ELISA) in culture when stimulated with killed *Staphylococcus aureus*. The bisbenzylisoquinoline alkaloid, tetrandrine inhibited the capacity of MNL and monocytes to produce TNF-α at a concentration range of 0·1 to 5 μg/ml. Tetrandrine may be potentially useful in the treatment of inflammatory diseases in which TNF-α plays a major role.

Keywords tetrandrine monocytes tumour necrosis factor-alpha

INTRODUCTION

Tetrandrine, the major alkaloid in 'Hanfangji' (the root of the creeper *Stephania tetrandra S Moore* of the Menispermaceal family) has been shown to retard and sometimes reverse the inflammatory and fibrotic lesions of pulmonary silicosis in clinical trials and in experimental silicosis in rats (Li *et al.*, 1981; Xu, Zou & Lih, 1983; Liu, Zou & Li, 1983). Evidence suggests that cells of the monocyte-macrophage series play a central role in the immunopathogenesis of silicosis, and silica-treated macrophages release factors which induce fibroblast proliferation and collagen synthesis (Allison, Harrington, Birbeck, 1966; Heppleston & Styles, 1967; Bateman, Emerson & Cole, 1982; Kulomen B, *et al.*, 1983; Bowden & Adamson, 1984). Two major macrophage cytokines which are likely to contribute as mediators in chronic inflammatory diseases such as silicosis are interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) which have similar biological activities including the stimulation of fibroblast growth (Schmidt *et al.*, 1984; Vilcek *et al.*, 1986). The anti-inflammatory effects of tetrandrine may be related to effects on production of these cytokines by macrophages. We now present evidence that tetradrine is an inhibitor of TNF-α production by human monocytes.

MATERIALS AND METHODS

Tetrandrine

Tetrandrine, a bisbenzylisoquinoline (M_r 622·73; empirical formula $C_{38}H_{42}O_6N_2$) (Seow, Li & Thong, 1986) was obtained from Yichang Pharmaceutical Company, Hupeh Province, People's Republic of China. A stock solution was made at a concentration of 2 mg/ml in ethanol. Dilutions of the drug were made in RPMI 1640 medium.

Leucocyte separation

Heparinized blood was layered onto Hypaque–Ficoll medium of density 1·114 (Ferrante & Thong, 1980) and centrifuged at 400 *g* for 30 min. The mononuclear leucocytes (MNL) in the top band (at the interface) were harvested. In addition, preparation of adherent mononuclear cells (containing predominantly monocytes) were made in 24-well cluster plates (Linbro, Flow Laboratories) as described previously (Seow *et al.*, 1989). To each well were added 2 ml of 1×10^6 MNL/ml and incubated at 37°C for 2 h. After incubation the non-adherent cells were removed and the adherent cells used for TNF-α production.

Stimulation of leucocytes for TNF-α production

To 1 ml of 2×10^6 MNL/ml was added 1 ml of 2×10^7 of *Staphylococcus aureus* in wells of 24-well cluster plate. *S. aureus* acted as a stimulus and was prepared as described previously (Ferrante *et al.*, 1987). The cells were cultured in RPMI 1640 medium supplemented with 2·5% **AB** heat-inactivated serum. Monocytes were similarly cultured and stimulated. Supernatants from these cell cultures were removed at the times indicated, frozen at −70°C and then assessed for levels of TNF-α.

Treatment of cells with tetrandrine

Cells were exposed to the indicated concentrations of tetrandrine 30 min prior to the addition of the stimulus, *S. aureus*, and cultured without removing exogenous tetrandrine. Preliminary studies had shown that the highest concentration of the diluent used did not affect TNF-α production by MNL and monocytes.

Correspondence: Dr A. Ferrante, Department of Immunology, Adelaide Children's Hospital, Adelaide, South Australia 5006.

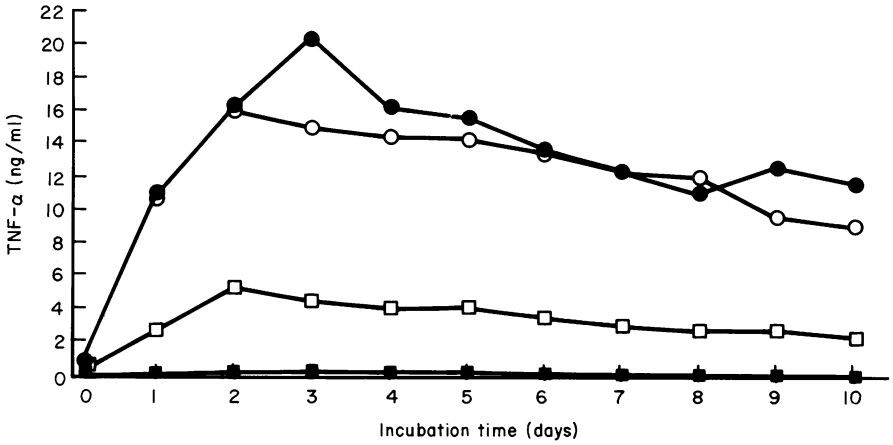

Fig. 1. Effects of tetrandrine on MNL TNF-α production. MNL were treated with 0 (●), 0·1 (○); 0·5 (□); or 1·0 (■) μg/ml tetrandrine and then stimulated with *S. aureus*. TNF-α was undetectable in the absence of a stimulus. Each point represents the mean of triplicate cultures.

Quantification of TNF-α

An ELISA was used for the quantification of human TNF-α in culture supernatants. The method was based on a monoclonal capture method similar to that used by Ferrante & Beard (1987) for the quantification of human IgG subclasses. Microtitre plates (Cooke, Dynatech Laboratories, Austria) were coated with an IgG fraction of goat anti-mouse IgG (Cappel, Malvern, PA). After washing, 50 μl of a mouse monoclonal antibody (kindly provided by Dr G. R. Adolf, Ernst-Boehringer Institut, Vienna, Austria) raised against recombinant human TNF-α (IgG1 isotype) were added to each well. After a further incubation and washing, 50 μl of either dilutions of standard recombinant TNFα or unknown (conditioned medium) were added to the wells. Following an 18-h incubation, 50 μl of a rabbit anti-TNF-α antisera were added and the plates incubated for 3 h. After a further washing step, 60 μl of donkey anti-rabbit IgG–horseradish-peroxidase conjugate (Amersham, Sydney) were added, and after a further 3-h incubation, 100 μl of substrate 2, 2′-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS Boehringer Mannheim, Sydney) were added. The colour reaction was allowed to proceed at 37°C for 45–60 min and the absorbance at 414 nm was measured in a Titertek Multiskan spectrophotometer (Flow Laboratories).

In these assays TNF-α was used for standard curves. The assays were run on numerous occasions and standard curves obtained were very similar. The sensitivity of the assay was 50–100 pg/ml. The assay was specific for TNF-α and it could not detect TNF-β (lymphotoxin), IL-1 and IL-2, and interferon gamma (IFN-γ).

Statistical analysis

The results were analysed by the two-tailed Student's *t*-test for paired data.

RESULTS

Results showed that both MNL and monocytes cultured without a stimulus did not produce detectable levels of TNF-α in every experiment conducted in this study.

Studies with MNL showed that *S. aureus* induced TNF-α production in ng/ml quantities, detectable within the first few hours of culture and reached maximum concentration within the first 3 days, and declining thereafter but still persisting after several days of culture (Fig. 1). Pretreatment of MNL with tetrandrine depressed the capacity of MNL to produce TNF-α (Fig. 1). At 1 μg/ml, tetrandrine completely inhibited TNF-α production. This inhibition persisted throughout the 10-day culture incubation period (Fig. 1).

Studies with monocytes stimulated with *S. aureus* showed production of levels of TNF-α similar to those produced by MNL (Fig. 2). Peak levels of TNF-α were also seen at approximately day 3. The concentration of cytokine in culture was maintained over the next few days. At 2·5 μg/ml, tetrandrine caused approximately 70% inhibition of *S. aureus*-induced TNF-α production by monocytes (Figs 2 and 3). Data with cells from three separate individuals are presented in Fig. 3. The inhibition was relatively consistent from individual to individual. The inhibition also persisted over the complete culture incubation period. Significant depression of TNF-α production was evident at 0·1 μg/ml of tetrandrine (Table 1) with a concentration related depression from 0·1 to 5 μg/ml (Table 1, Fig. 3).

Using trypan blue dye exclusion as a criterion for cell viability it was found that concentrations of tetrandrine used in this study (up to 5 μg/ml; and examined at various time points, up to 3 days of culture) were not toxic to the leucocytes.

DISCUSSION

Data presented show that tetrandrine is a potent inhibitor of TNF-α production by human monocytes. Previously we have shown that production of another cytokine, IL-1, by human monocytes is also inhibited by this drug (Seow, Thong & Ferrante, 1989). Although TNF-α and IL-1 differ in some important properties (Seow *et al.*, 1987), these cytokines share many biological properties (Rosenblum & Donato, 1989), and the inhibitory effects of the alkaloid on monokine production may be one of its principle mode of action in relation to the anti-inflammatory effects of tetrandrine.

The effects of the drug on the macrophage appears to be highly selective. At concentrations of 10 μg/ml it had no effect on the monocyte degranulation response and the oxygen-

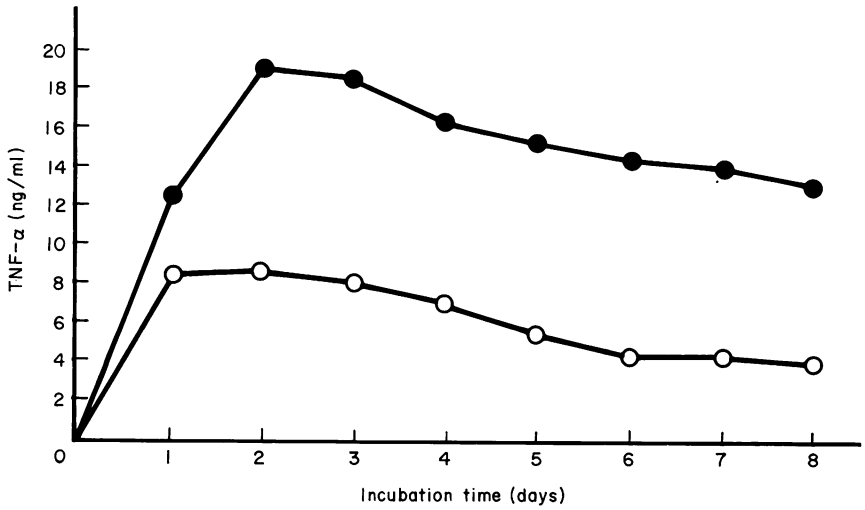

Fig. 2. Effects of tetrandrine on TNF-α production by monocytes. Monocytes were treated with either dilutent (●) or 2·5 μg/ml tetrandrine (○).

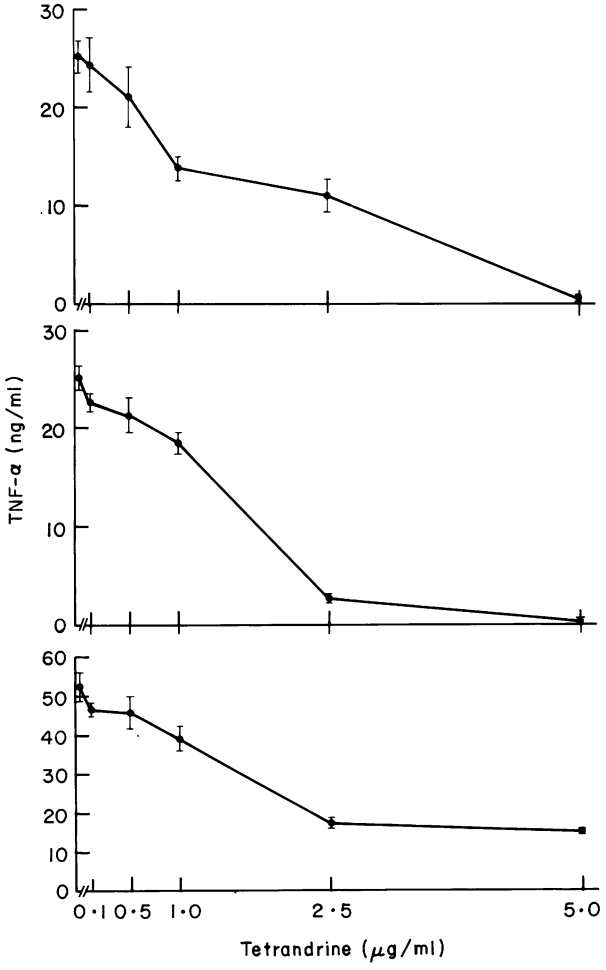

Fig. 3. Effect of different tetrandrine concentrations on TNF-α production by monocytes from three donors. Measurements were made on day 3 of culture. Each panel represents results (mean ± s.d.) from one of three individuals.

Table 1. Effects of different concentrations of tetrandrine on TNF-α production by monocytes

Tetrandrine concentration (μg/ml)	TNF-α production (± control response)*
0·1	92·5 ± 7·2†
0·5	87·3 ± 9·0†
1·0	67·9 ± 11·3§
2·5	28·8 ± 15·1§
5·0	10·3 ± 13·3§

* Results are presented as mean ± s.d. of results obtained with cells from three individuals (shown in Fig. 3) of measurments made on day 3 of culture.
† $P < 0.01$; § $P < 0.001$.

dependent respiratory burst (Seow *et al.*, 1989). However, the drug did inhibit monocyte adherence and chemotaxis (Seow *et al.*, 1989) and was shown to be a superoxide scavenger (Seow *et al.*, 1988b, 1989). Similar effects were observed for human neutrophils (Seow *et al.*, 1986, 1988b).

Prostaglandin E_2 (PGE_2) regulates production of TNF-α by macrophages (Renz *et al.*, 1988). However, since tetrandrine inhibits prostaglandin production (Teh, Seow & Thong, 1990), it is unlikely that the inhibition of TNF-α production is related to enhanced production of PGE_2. Activation of protein kinase appears to be important in stimulation of leucocytes for TNF production (Cuturi *et al.*, 1987). Following ligand binding to leucocyte receptors a phosphodesterase is activated which converts polyphosphatidylinositol to inositol-1,4,5-triphosphate and diaglycerol. These agents promote the activation of protein kinase C. Our recent studies have shown that tetrandrine depressed the formation of inositol phosphate, calcium flux and protein kinase C translocation in leucocytes (Ioannoni *et al.*, 1989). It is plausible that tetrandrine may inhibit TNF-α production predominantly by affecting activation of protein kinase C. Other mechanisms could also involve decreased

stability of TNF-α mRNA similar to effects of glucocorticoids on IL-1β mRNA (Lee *et al.*, 1988).

Previously it has been demonstrated that tetrandrine inhibits NK cell cytotoxicity against tumour cells (Seow *et al.*, 1988a). It is therefore tempting to speculate that inhibition of NK cell cytotoxicity may be related to a depression in TNF-α production by NK cells since these cells are also known to produce TNF-α. The inhibitory properties of tetrandrine on TNF-α and IL-1 production by macrophages is most likely the major mechanism of its therapeutic efficacy in silicosis. Both IL-1 and TNF-α could contribute to the development of inflammatory lesions in silicosis by stimulating fibroblast proliferation.

TNF-α amd IL-1 are multifaceted peptide hormones with many related activities pertinent to a wide variety of inflammatory disease, and also in endotoxic shock. It is thus conceivable that this drug or its derivatives may be used as useful broad spectrum anti-inflammatory drugs (Li *et al.*, 1989). This optimistic view is supported by other findings which have shown its inhibitory effects on other inflammatory mediators such as mast cell histamine release (Teh *et al.*, 1988), platelet-activating factor-induced platelet aggregation (Teh *et al.*, 1989) and production by leucocytes of prostaglandins and leucotrienes (Teh *et al.*, 1990).

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