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Benzydamine protection in a mouse model of endotoxemia

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Abstract. *Objective*: Previous studies have shown that benzydamine (40 mg/kg s.c.) is able to inhibit tumor necrosis factor (TNF) production and to reduce mouse lethality when administered before or concomitantly with LPS. The present study was designed to further investigate benzydamine activity against LPS-induced toxicity in terms of potency and therapeutic effects.
Methods: Female Balb/c mice were used. A dose-response curve of animal lethality versus endotoxin dose was performed (LD_{50} = 45 μg/mouse). Therapeutic effects were studied selecting the dose of LPS to achieve an LD_{100} (160 μg/mouse). Mortality was assessed daily and mice were followed for 8 days. The potential mode of action of therapeutically administered benzydamine was also investigated. TNFα and IL-1β levels were measured, at 5 h after LPS injection, both in sera and in lungs. Moreover, the drug was assayed in a TNF-dependent cytoxicity test.
Results: Benzydamine, administered at 20 mg/kg s.c. simultaneously with the endotoxin, significantly increased LPS LD_{50} up to 230 μg/mouse ($p < 0.05$). Moreover, the drug significantly protected mice against LPS-induced lethality when administered either 30 min or 4 h after endotoxin injection ($p < 0.001$). Benzydamine, therapeutically administered at 20 mg/kg s.c., significantly reduced TNFα and IL-1β production induced by LPS both in serum and lungs and it was shown to inhibit TNF-dependent cytoxicity on L929 cells.
Conclusions: These results clearly demonstrate the therapeutic activity of benzydamine in a simple model of endotoxic shock. Available data confirm the potential role of benzydamine as an anti-cytokine agent and provide suggestions for novel therapeutic applications of this anti-inflammatory drug.

Key words: Endotoxic shock – LPS – Mice – Inflammatory cytokines – TNFα

Introduction

Severe bacterial infections can result in profound physiological changes such as hypotension, fever, tissue necrosis, organ dysfunction and, ultimately, death. Lipopolysaccharide (LPS) is a component of endotoxin released from gram-negative bacteria and is largely responsible for the morbidity and mortality associated with infections by these microorganisms. Consequently, the injection of appropriate doses of LPS in experimental animals can produce effects that are typical of the septic shock syndrome, thus providing a simple animal model for this inflammatory reaction [1,2]. LPS-induced toxicity appears to be related to an overproduction of cytokine such as tumor necrosis factor (TNF) and/or interleukin-1 (IL-1), since animals can be protected from endotoxic shock by neutralizing these inflammation mediators [3,4].

Benzydamine, or N,N-dimethyl-3-[[1-(phenylmethyl)-1H-indazol-3-y1]oxy]-1-propanamine, is a non-steroidal anti-inflammatory drug mainly devoid of activity on arachidonic acid metabolism and endowed with local anaesthetic and analgesic properties [5]. The drug is extensively used in clinical practice for the topical treatment of inflammatory conditions [6]. Recent studies have shown that benzydamine reduces TNF production and protects mice from LPS-induced mortality [7].

The aim of the present study was to further investigate the protective activity of benzydamine against LPS-induced toxicity in terms of potency and therapeutic effects using a mouse model of endotoxemia.

Materials and methods

Endotoxin-induced shock

Balb/c female mice (18–20 g) obtained from Charles River Italia (Calco, Como, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985). Shock was induced by an intraperitoneal injection of Lipopolysaccharide (LPS) B Escherichia coli 055:B5 (Difco Laboratories, Detroit, MI, USA) suspension in sterile saline. Mice were injected with 200 μl containing doses of LPS ranging from 5 to 640 μg. A dose-response curve of animal mortality versus endotoxin dose was performed in order to calculate LD_{50} of the specific batch of LPS used (45 μg/mouse).

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Therapeutic effects were studied selecting the dose of LPS to achieve LD_{100} (160 μg/mouse). Benzydamine hydrochloride (ACRAF, Aprilia, Italy) was dissolved in sterile saline and administered subcutaneously (10 ml/kg body weight) at 20 or 10 mg/kg. Drug administration was performed simultaneously to LPS injection for potency evaluation, whereas for therapeutic studies benzydamine was given either 30 min or 4 h after endotoxin injection. In all experiments mortality was assessed daily and mice were followed for 8 days.

Cytokine determination

Cytokine measurements were performed using commercially available immunoenzymatic kits (Genzyme, Cambridge, MA, USA) according to the manufacturer's instructions. TNFα and IL-1β levels were evaluated, at 5 h from the injection of an LD_{100} dose of LPS (160 μg/mouse), both in sera and in lungs obtained from control mice and from mice treated with benzydamine (20 mg/kg s.c.) 4 h after endotoxin injection. Blood was taken from the retroorbital plexus after which mice were sacrificed and lungs removed immediately, weighed and frozen in liquid nitrogen and kept at -80 °C until analysis. Lung cytokine determination was carried out on homogenate prepared in a blender by diluting the tissue in cold PBS buffer (1:3, w:v) containing 0.4 mM phenylmethylsulphonyl-fluoride (Sigma, St. Louis, MO, USA).

TNF-induced cytotoxicity assay

The assay was performed using the TNF-sensitive L929 murine fibrosarcoma cell line (ATCC, Rockville, MD, USA) [8]. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 1% penicillin-streptomicin, 2% L-glutamine and 20 mM Hepes (Flow Laboratories, Rickmansworth, UK). 1×10^4 L929 cells/well were plated in flat-bottom microtiter plates; 24 h later the cells were treated with 100 μl/well of Actinomicin D (2 mg/ml) for 2 h. Subsequently 50 μl of medium containing 1 U/ml of murine recombinant TNF (Genzyme, Cambridge, MA, USA) and 50 μl of benzydamine solution or vehicle were added. The drug was tested at concentrations ranging from 12.5 to 100 μM. Control wells were also prepared by adding benzydamine in the absence of TNF in order to exclude a product-related toxicity. After 24 h the medium was removed, surviving cells were stained with the crystal violet dye and optical density values were measured at 540 nm. Three separate experiments were performed. Percentage of cytotoxicity was calculated using the following formula: $100(a - b)/a$, where a and b represent the mean absorbances of triplicate wells with culture medium alone and test sample, respectively.

Statistical analysis

Statistical analyses were performed using the Litchfield & Wilcoxon test for dose-response curve calculations and comparisons, and the $\chi 2$ test for therapeutic activity. Cytokine levels were compared using Student's t-test for unpaired data. Results of TNF-induced cytotoxicity assay were analyzed by the Dunnett's test.

Results

Effects on endotoxin-induced lethality

Dose-response curves of animal lethality versus endotoxin dose are shown in Figure 1. Groups of at least 10 Balb/c female mice were injected intraperitoneally with LPS, at doses ranging between 5 and 640 μg/mouse, with or without simultaneous administration of benzydamine at 20 mg/kg s.c.. Benzydamine significantly increased LPS LD_{50} from 45 μg/mouse up to 230 μg/mouse ($p < 0.05$).

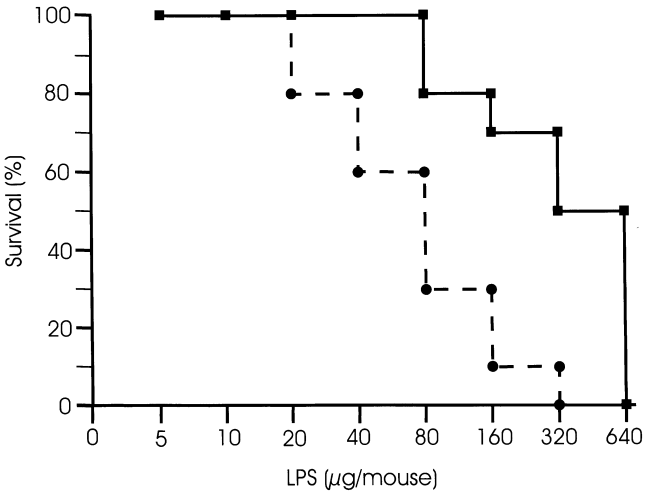

Fig. 1. Dose-response curves of mice lethality versus endotoxin dose. Mice were treated i.p. with LPS with (■) or without (●) a simultaneous injection of benzydamine (20 mg/kg s.c.) and mortality was assessed daily and expressed as a percentage (at least 10 mice/group). Animals were followed for up to 8 days. Calculated LD_{50}: LPS alone 45 μg/mouse vs. LPS with benzydamine 230 μg/mouse ($p < 0.05$ by Litchfield & Wilcoxon test).

Table 1. Therapeutic effects of benzydamine on endotoxin-induced lethality.

Benzydamine (mg/kg s.c.)	No. survivors/total mice	
	30 min	4 h
0	1/60	1/60
10	7/20*	3/20
20	40/50*	22/50*

Mice were treated with LPS (160 μg/mouse, i.p.) with or without benzydamine (30 min or 4 h after endotoxin injection) and survival was assessed daily. Mice were followed up to 8 days.
* $p < 0.001$ vs. LPS alone by $\chi 2$ test.

Therapeutic effects obtained administering benzydamine 30 min or 4 h after intraperitoneal injection of the dose of LPS to achieve LD_{100} (160 μg/mouse) are shown in Table 1. The drug, at 20 mg/kg s.c., was able to significantly protect mice against LPS-induced lethality when administered either at 30 min or at 4 h ($p < 0.001$). Benzydamine at 10 mg/kg s.c. provided partial protection with a significant reduction of endotoxin lethality only when administered 30 min after LPS injection ($p < 0.001$).

In all experiments, animals were followed for 8 days and neither further deaths nor surviving, unhealthy mice were observed after this period.

Effects on cytokine production

Benzydamine, given 4 h after endotoxin injection at the dose of 20 mg/kg s.c., was able to significantly reduce TNFα and IL-1β serum levels as measured 5 h after LPS administration (Table 2). Moreover, measurement of cytokines in the homogenate of lungs from the same animals showed a significant inhibition of TNF but not of IL-1 production

Table 2. Effects of benzydamine on endotoxin-induced serum TNF and IL-1.

Treatment	TNF (ng/ml)	IL-1 (pg/ml)
LPS	3.2 ± 0.59	148.8 ± 136.39
Benzydamine	2.0 ± 0.14*	47.3 ± 46.34*

Mice were treated with LPS (160 μg/mouse, i.p.) with or without benzydamine (20 mg/kg s.c., 4 h after endotoxin injection) and cytokines determined at 5 h from LPS administration. Data are mean ± SD (10 mice/group).
* $p < 0.05$ vs. LPS alone by Student's t-test.

Table 3. Effects of benzydamine on endotoxin-induced TNF and IL-1 production in the lungs.

Treatment	TNF (ng/g)	IL-1 (ng/g)
LPS	2.1 ± 1.10	11.8 ± 1.61
Benzydamine	0.8 ± 0.84*	9.7 ± 3.71

Mice were treated with LPS (160 μg/mouse, i.p.) with or without benzydamine (20 mg/kg s.c., 4 h after endotoxin injection) and cytokines determined on lung homogenate at 5 h from LPS administration. Data are mean ± SD (10 mice/group).
* $p < 0.05$ vs. LPS alone by Student's t-test.

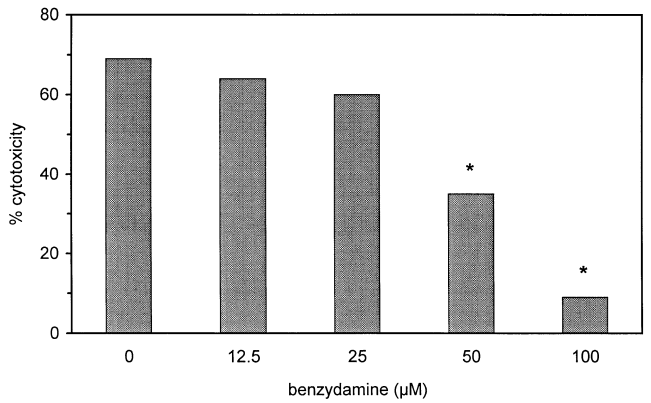

Fig. 2. Effects of benzydamine on TNF-induced (1 μg/ml) cytotoxicity in TNF-sensitive L929 cell line. Results reported represent the means of the three experiments performed. * $p < 0.01$ vs. TNF alone by Dunnett's test.

(Table 3); nevertheless, a 50% reduction in IL-1 lung content was observed in 3 out of 10 mice examined.

Effects on TNF-induced cytotoxicity

Figure 2 shows the effects of different concentrations of benzydamine on TNF-induced cytotoxicity in the TNF-sensitive L929 cell line. Three different experiments were performed which all gave similar results. TNF alone induced death in about 70% of the plated cells, while the presence of benzydamine dramatically decreased the number of killed target cells. The protective effect was dose-dependent and statistically significant at 50 and 100 μM ($p < 0.01$).

Discussion

Sepsis continues to be one of the most common cause of death in intensive care units, despite the use of specific antibiotics, careful monitoring and aggressive operative intervention. Most of the physiological effects of bacterial infection are mediated by a complex interaction of proinflammatory mediators such as cytokines, lipid metabolites and oxygen free radicals which ultimately cause organ dysfunction. Among cytokines, tumor necrosis factor (TNF) is frequently considered as a central mediator in the sepsis syndrome and it is thought to play a pivotal role in the pathogenesis of multiple organ failure [9,10]. In fact, intravenous administration of TNF causes tissue damage and shock, as well as intravascular injections of endotoxin or gram-negative bacteria which induce similar lesions, probably by a mechanism mediated by a dramatic increase in TNF serum concentration. Consequently, many attempts have been made to find anti-cytokine agents of some clinical relevance for the treatment of patients with sepsis [3,4,11–13] but so far trials have been unsuccessful in reducing mortality significantly [14].

Previous findings have shown that benzydamine, a non-steroidal anti-inflammatory agent devoid of effects on arachidonate metabolism, consistently inhibits the production of TNF in vitro and in vivo [5–7]. On this basis, the present study was designed to verify the potency and the therapeutic activity of this molecule in a simple animal model of endotoxin-induced shock. The results obtained by simultaneous administration of benzydamine with appropriate doses of LPS confirm the previously observed protective activity of the molecule in this model and, in terms of potency, show that the product can increase the LD_{50} more than 5-fold with respect to saline-treated animals. In order to explore the therapeutic activity of benzydamine, the product was then administered up to 4 h after the injection of an amount of LPS sufficient to give a 100% mortality; the dose of 20 mg/kg benzydamine significantly protected mice when the drug was administered both at 30 min or 4 h after LPS, whereas at a lower dose (10 mg/kg), the product exerted its effect only when given 30 min after endotoxin injection.

Since peak TNF and IL-1 blood levels after LPS injections are reached within 1 h and 3 h, respectively [15], the protective effects on mortality observed with benzydamine given prophylactically can be reasonably explained by the capacity of the drug to decrease the release of these inflammatory cytokines from macrophages [7]. Such a reduction, however, can hardly explain by itself the improvement in survival obtained by administration of benzydamine as late as 4 h after LPS injection. Nevertheless, the paracrine function of TNF and the events at the cellular or tissue level associated with the release and activity of this cytokine should be considered. In fact, Hadjiminas et al. have detected significantly augmented TNF mRNA levels in various tissues (liver, spleen, lung) up to 6 h after LPS injection [10]. Moreover, as the IL-1 level is maximal at 3 h after LPS injection and remains fairly elevated for several hours [15], the inhibiting activity of benzydamine on IL-1 release [7] could be responsible partially for its therapeutic action. In line with this view, additional studies were designed to provide a possible explanation for the protective

effects exerted by benzydamine when administered as late as 4 h after endotoxin injection.

In a series of experiments, the capacity of the product to reduce serum cytokine concentration on therapeutic administration was examined. The results demonstrate that several hours after LPS injection TNF and IL-1β blood levels are still elevated in comparison to control mice and that the drug, administered at 4 h from endotoxin, can significantly lower both TNF and IL-1β serum content measured 5 h after LPS treatment. Furthermore, since an elevation of TNF and IL-1 concentration has been reported in several organs, such as lungs, liver or spleen, in the LPS-induced multi-organ failure syndrome [10], the effects of benzydamine on lung cytokine concentration were examined. The therapeutic administration of the drug strikingly reduced TNF lung content measured 5 h after LPS administration and also showed a partial effect on IL-1β.

TNF is a protein which possesses cytotoxic and cytostatic activity against a large variety of tumors and in particular conditions, such as in response to endotoxin, is also cytotoxic to normal tissues [3,16,17]. In order to better elucidate the mechanisms by which benzydamine may provide protection in this endotoxic shock model when administered 4 h after LPS, the activity of the product in an in vitro TNF-dependent cytotoxicity test was analyzed. Such experiments showed that benzydamine is able to protect L929 fibroblast cells from TNF-mediated lysis at concentrations which are comparable to those observed both in plasma and in inflamed tissues following systemic administration of the product [18,19].

The overall results confirm and extend our previous observations on the ability of the product to inhibit TNF-production after stimulation with LPS. Benzydamine, in fact, besides reducing TNF and IL-1 concentrations in the general circulation, was found to exert a similar action also in specific areas, such as the lungs, which are the preferential target organ in the shock syndrome. In addition, the in vitro experiments on TNF-mediated cytoxicity show that the product is able to protect the cells from cytotoxic effects due to the massive TNF production that takes place in endotoxic shock. However, it cannot be excluded that, besides activity against cytokine formation and/or action, the therapeutic efficacy of benzydamine in this model may be due to additional mechanisms.

It is worth stressing that although many different compounds have been reported to decrease LPS-dependent inflammatory cytokine production both in vitro and in vivo and/or to reduce mortality of endotoxin-treated animals [11–13,20–22], to the best of our knowledge, no drugs are presently available which exert a therapeutic effect in this experimental model. Hence, further work needs to be carried out to better define the mode of action and the potential role of benzydamine as an anti-cytokine agent. In this respect, the results presented suggest therapeutic applications of this anti-inflammatory drug in diseases in which cytokines play an etiopathogenetic or an amplification role.

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