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Benzydamine inhibits the release of tumor necrosis factor-α and monocyte chemotactic protein-1 by *Candida albicans*-stimulated human peripheral blood cells

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Abstract Benzydamine is a non-steroidal antiinflammatory drug, devoid of activity on arachidonic acid metabolism, which is extensively used as a topical drug in inflammatory conditions, particularly for the treatment of bacterial vaginosis and *Candida albicans*-sustained vaginitis. In the present study the effects of benzydamine on the production of several inflammatory cytokines were examined in cultures of *Candida albicans*-stimulated human mononuclear cells. Benzydamine (6.25–50 μM) inhibited *Candida*-induced tumor necrosis factor-α and, to a lesser extent, interleukin-1β production, whereas it did not affect interleukin-6 release. Benzydamine also blocked monocyte chemotactic protein-1 secretion, but it did not affect interleukin-8 production. Unlike benzydamine, ibuprofen and naproxen, two non-steroidal antiinflammatory drugs also used topically, were unable to suppress inflammatory lymphokine production from *Candida*-activated mononuclear cells. These data suggest that benzydamine may be effective in local *Candida* infections at least in part by suppressing inflammatory cytokine and monokine production in the vaginal mucosa and consequently decreasing their levels in vaginal secretions.

Key words Antiinflammatory drug · Interleukins · Chemokines · *Candida albicans* · Vaginitis

Introduction

Benzydamine, or *N,N*-dimethyl-3-[[1-(phenylmethyl)-1H-indazol-3-yl]oxy]-1-propanamine, is a non-steroidal antiinflammatory drug (NSAID) which is devoid of activity on arachidonic acid metabolism and has local anesthetic and analgesic properties [1]. Benzydamine is extensively used in clinical practice for the topical treatment of inflammatory conditions [2]; in particular, benzydamine is highly effective in the treatment of bacterial vaginosis and vaginitis, including vaginal inflammatory conditions sustained by *Candida albicans* yeasts [3].

C. albicans is an opportunistic pathogen which is part of the normal flora of the oral cavity and of the lower gastrointestinal and urogenital tracts of humans. Changes in the vaginal environment enable *C. albicans* to outgrow the normal flora and cause vaginitis. The effector cells responsible for the control of *C. albicans* growth and for anti-*Candida* responses are primarily polymorphonuclear neutrophils and monocyte/macrophages [4]; these cells act not only by phagocytosis and intracellular killing, but also by releasing several mediators of the inflammatory response, such as eicosanoids and cytokines [5]. Moreover, human natural killer cells, resident macrophages and peripheral blood monocytes have been shown to secrete tumor necrosis factor (TNFα) when interacting with *C. albicans* yeast cells [6–8] or even with its cell wall polysaccharide mannan [9].

Benzydamine has been recently shown to have cytokine suppressor activity, since it can inhibit the in vitro production of TNFα and, to a lesser extent, of interleukin-1β (IL-1β) by human and murine mononuclear cells exposed to various inducers; in addition, in vivo administration of benzydamine consistently reduces peak blood levels of TNFα and IL-1β in lipopolysaccharide (LPS)-treated mice [10].

Such observations led us to the hypothesis that one of the mechanisms by which benzydamine may be effective in the treatment of *Candida*-sustained vaginal inflammation is by normalizing the production of inflammatory cytokines and chemokines in vaginal mucosa and secretions. The present study was therefore undertaken to assess the effects of benzydamine and other NSAIDs on the secretion of inflammatory cytokines (TNFα, IL-1β, and IL-6) and chemokines [IL-8 and monocyte chemotactic

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protein 1 (MCP-1)] by human leukocytes stimulated with *C. albicans* yeasts.

Materials and methods

C. albicans preparation

C. albicans was obtained from the American Type Culture Collection (Rockville, USA). The yeast was grown in Sabouraud dextrose broth (Difco, Detroit, USA) at 35 °C until the stationary phase of growth was reached. Cells were then harvested by centrifugation (2,000 *g* for 15 min), washed twice with sterile saline solution and once with sterile distilled water. *C. albicans* was resuspended in 50 ml of sterile water and killed by heating in boiling water for 4 h. After inactivation, the yeast was lyophilized: 1 mg of dry material corresponded to approximately 5×10^7 yeast cells [11].

Possible pyrogen contamination was investigated by testing the lyophilized *Candida* preparation with a Limulus assay (LAL Test QCL-1000, BioWhittaker, Walkersville, USA). The minimal sensitivity of the test is 0.1 endotoxin units. The preparation was found to be pyrogen free.

Peripheral blood mononuclear cell preparation and treatment

Buffy coats from blood donations (Centro Trasfusionale, Ospedale Sacco, Milan, Italy) were used as a source of peripheral blood mononuclear cells (PBMCs) which were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) gradient centrifugation; 2×10^5 cells in 0.2 ml of RPMI containing 1% fetal calf serum were cultured in the presence of various concentrations of test drugs and/or *Candida* (4×10^7 cells/ml) in polypropylene round-bottomed 96-well plates for 24 h; this incubation time was chosen, as optimal cytokine induction by *Candida* has been observed after a 24-h incubation ([12]; Sironi et al., unpublished observations). The supernatants were then collected, spun and frozen at −20 °C until use. Drug toxic effects on cell viability were analyzed and excluded by trypan blue staining.

Benzydamine hydrochloride was kindly provided by A.C.R.A.F. S.p.A. (Ancona, Italy). Naproxen (6-methoxy-α-methylnaphthaleneacetic acid) and ibuprofen [α-methyl-4-(2-methylpropyl)benzeneacetic acid] were obtained from Sigma (St. Louis, USA).

Cytokine determination

Human TNFα was detected using a sandwich immunoenzymatic assay as previously described [10]. The sensitivity of the test is 600 pg/ml. Human IL-1β was measured by a commercially available immunoenzymatic kit (Amersham, Salisbury, UK), according to the manufacturer's instructions; the test sensitivity is reported to be 1 pg/ml.

Human IL-6 was measured as hybridoma growth factor activity on the 7TD1 cell line, according to Sironi et al. [13], using human recombinant IL-6 as a reference standard. Human MCP-1 was determined by a specific monoclonal sandwich ELISA, as detailed elsewhere [14]. The sensitivity of the assay is 100 pg/ml. Human IL-8 levels were measured as described by Bernasconi et al. [15]. All measurements were performed in duplicate or triplicate.

Statistical analysis

The amounts of cytokines released by *Candida*-stimulated PBMCs (baseline values) and those induced by *C. albicans* in the presence of graded doses of benzydamine were recorded for each test. Data were analyzed using SPSS/PC version 4.0. The MANOVA procedure was utilized on logarithmic-transformed data to statistically evaluate the ratio of cytokine measured at each benzydamine concentration versus baseline values and the relative standard deviations.

For each single donor the differences among cytokine levels obtained in the presence of various doses of benzydamine and those detemined in untreated control samples were statistically analyzed by Dunnett's test.

Results

Effects of benzydamine on *Candida*-induced cytokine release

Besides inducing significant production of TNFα as expected (5–55 ng/ml), *C. albicans* caused mononuclear cells to secrete other inflammatory cytokines, namely IL-1β and IL-6; the amount of IL-1β released was between 0.05 and 2.5 ng/ml, whereas the IL-6 content was in the range of 250–6,000 U/ml. Since these cytokines, as well as TNFα, may play an important role in patients with vaginal inflammatory *Candida*-dependent conditions, the effects of benzydamine on *Candida*-induced TNFα, IL-1β and IL-6 secretion were examined in human mononuclear cells from at least five different donors. PBMCs were exposed to four different concentrations of the drug (50, 25, 12.5, and 6.25 μM) in the presence of heat-inactivated *C. albicans* cells (4×10^7/ml) for 24 h; in the experiments measuring TNFα release the 6.25 μM benzydamine concentration was not used. Table 1 shows a typical experiment in which PBMCs from a normal donor were examined for TNFα,

Table 1 Effect of benzydamine on cytokine and chemokine production by peripheral blood mononuclear cells (ND not determined)[a]

Benzydamine	*Candida albicans*-induced production of				
	TNFα (ng/ml)	IL-1β (pg/ml)	IL-6 (U/ml)	IL-8 (ng/ml)	MCP-1 (ng/ml)
Medium	7.6±1.6	73.01±6.46	419±105	2.99±0.22	1.56±0.21
6.25 μM	ND	51.82±6.02	354±73	2.48±0.26	1.14±0.24*
12.5 μM	6.4±0.9	47.20±3.24*	316±91	2.78±0.31	1.03±0.22**
25.0 μM	4.9±0.5**	39.52±4.19**	656±80**	2.57±0.12	0.72±0.06**
50.0 μM	1.9±0.2**	66.20±5.66	353±307	2.77±0.29	0.64±0.05**

* $P<0.05$ vs. medium; ** $P<0.01$ vs. medium by Dunnett's test

[a] Results are from one representative normal subject of the five (TNFα, IL-1β, IL-6 and IL-8) or six (MCP-1) examined

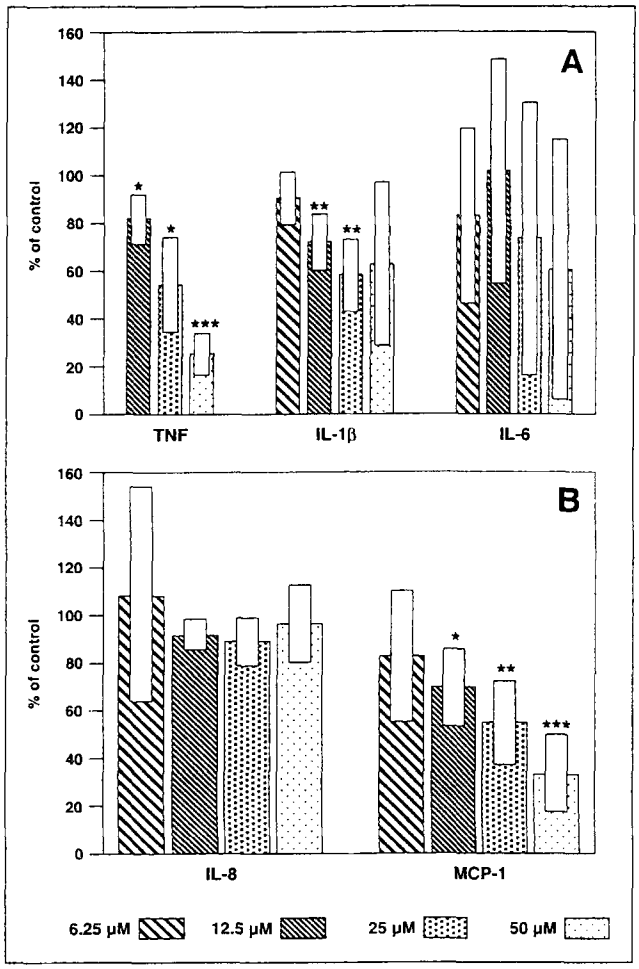

Fig. 1 Effect of benzydamine on inflammatory cytokine and chemokine production by peripheral blood mononuclear cells (PBMCs) in all the examined subjects. Cytokines (**A**) and chemokines (**B**) were measured in the supernatants of 2×10^5 PBMC/0.2 ml stimulated with 4×10^7 *Candida albicans*/ml and cultured with medium alone or with graded concentrations of benzydamine for 24 h. * $P<0.05$, ** $P<0.01$, and *** $P<0.005$ by MANOVA

IL-1β and IL-6 production in response to *C. albicans* in the presence or absence of graded concentrations of benzydamine. In this subject benzydamine markedly inhibited TNFα and IL-1β production, while IL-6 release was not affected.

As summarized in Fig. 1A, benzydamine caused a dose-dependent reduction in TNFα and, to a lesser extent, IL-1β release. The drug exerted a statistically significant inhibition on TNFα production at all the concentrations tested (12.5–50 μM) compared with *Candida*-stimulated controls; this inhibition reached 50% of the control at the highest dose of benzydamine in all subjects tested. A statistically significant reduction in IL-1β release was observed only at benzydamine concentrations of 12.5 and 25 μM; a significant decrease ($P<0.01$ by Dunnett's test) of IL-1β production in the presence of 50 μM benzydamine was observed in only three of the five donors. Benzydamine did not modify IL-6 release from PBMCs stimulated with *C. albicans*; however, in two of the five donors a slight decrease ($P<0.05$ by Dunnett's test) in IL-6 concentration was observed at the highest dose (50 μM) of benzydamine.

Effects of benzydamine on chemokines

Culture supernatants collected from *Candida*-stimulated PBMCs in the presence of graded doses of benzydamine (6.25–50 μM) were also examined for the presence of the chemokines MCP-1 and IL-8 which are involved in monocyte/macrophage and polymorphonuclear cell recruitment into inflammatory sites [16]. *C. albicans* induced the release of both MCP-1 (1–10 ng/ml) and IL-8 (3–15 ng/ml) from PBMCs. Table 1 shows the results of a typical experiment where the release of IL-8 and MCP-1 from *Candida*-activated PBMCs of a normal donor was assessed in the presence of different doses of benzydamine. In this donor benzydamine reduced MCP-1 but not IL-6 release. The effects of benzydamine on chemokine production from PBMCs in response to *C. albicans* stimulation are summarized in Fig. 1B. The drug significantly reduces MCP-1 production in a dose-dependent fashion starting from the 12.5 μM concentration; this was consistently observed in all donors, for three of whom a significant inhibition was present at a benzydamine concentration of 6.25 μM ($P<0.01$ by Dunnett's test). No effects of benzydamine on *Candida*-induced IL-8 release were observed in any donor.

Comparison of benzydamine activity with other NSAIDs

Naproxen and ibuprofen are NSAIDs which are commonly prescribed as vaginal washes for the treatment of inflammatory vaginal conditions. Figure 2 shows the effects of different concentrations of these drugs (12.5–50 μM) on the production of TNFα (A) and MCP-1 (B), in comparison with benzydamine at the same concentrations; three donors were examined for TNF-α inhibition and two for MCP-1. Whereas the inhibitory effect of benzydamine on TNFα and MCP-1 production was confirmed, neither naproxen nor ibuprofen reduced the amount of these cytokines secreted from *Candida*-treated human cells; moreover, no difference was observed among the various donors. Table 2 shows the results obtained by stimulating the PBMCs from one representative normal donor with inactivated *C. albicans* in the presence of graded doses of the different drugs.

Discussion

The results of this study confirm previous observations that benzydamine is able to decrease the TNFα and, to a lesser

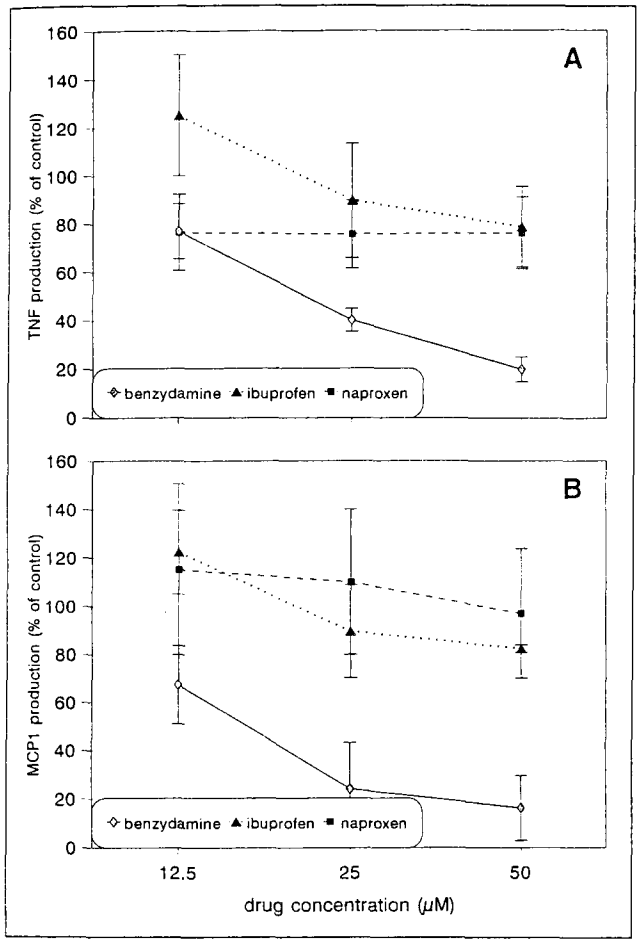

Fig. 2 Effect of benzydamine, ibuprofen and naproxen on TNFα and MCP-1 production by *Candida*-stimulated PBMCs in all the examined subjects. **A** TNFα; **B** MCP-1

Table 2 Activity of benzydamine and different non-steroidal anti-inflammatory drugs on TNFα and MCP-1 production[a]

Treatment		*C. albicans*-induced production of	
		TNFα (ng/ml)	MCP-1 (ng/ml)
Medium		25.4± 8.1	3.34±0.70
Benzydamine	12.5 μM	20.3± 4.4	2.64±0.42
	25 μM	10.9± 1.2*	0.36±0.05*
	50 μM	6.2± 0.5*	0.23±0.04*
Ibuprofen	12.5 μM	35.6± 5.0	3.68±0.30
	25 μM	26.0±14.2	2.54±0.33
	50 μM	23.3±13.4	2.72±0.39
Naproxen	12.5 μM	22.4±12.5	2.78±0.26
	25 μM	21.1±10.6	2.97±0.75
	50 μM	22.0±10.9	2.61±0.36

* $P<0.01$ vs. medium by Dunnett's test

[a] Results are from one representative normal subject of the three (TNFα) or two (MCP-1) examined

extent, IL-1β secretion induced by different stimuli [10]: benzydamine inhibited TNFα and IL-1β release by human peripheral leukocytes stimulated with heat-inactivated *C. albicans*. This effect was dose dependent and statistically significant at 12.5, 25 and 50 μM benzydamine for TNFα and 12.5 and 25 μM for IL-1β. These findings are particularly interesting as benzydamine is known to be therapeutically effective in *C. albicans*-dependent vaginal inflammatory states [3]. The present data suggest that one of the mechanisms by which benzydamine may exert its antiinflammatory activity is the reduction of inflammatory cytokine levels in the vaginal mucosa and secretions.

In the present study it was also shown that the *Candida*-induced production of the chemokine MCP-1 is significantly reduced by benzydamine, starting at a concentration of 12.5 μM. This is particularly significant as vaginal secretions of patients with bacterial vaginosis and *Candida*-induced vaginitis contain large numbers of leukocytes [17]. As inflammatory cell recruitment is partially mediated by soluble chemokines [16], the inhibitory activity of benzydamine on the release of MCP-1 may have a pivotal role in the therapeutic effects of the product.

Unlike TNFα, IL-1β and MCP-1, IL-6 and -8 production was not affected by benzydamine. These results are in agreement with previously published data [10], which indicated that benzydamine could not modulate IL-6 secretion by cells stimulated with LPS or inactivated streptococci. It is worth noting that such differential effects on TNFα, IL-1β and MCP-1 on the one hand and on IL-6 and -8 on the other could provide a key to the understanding of the mechanism of action of benzydamine at the molecular level.

Unlike benzydamine, the arylpropionic acids ibuprofen and naproxen, commonly used as antiinflammatory drugs for the treatment of vaginal inflammations, did not inhibit cytokine release. It is tempting to speculate that the anti-inflammatory clinical efficacy of benzydamine depends at least in part on its capacity to block cytokine production.

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