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Benzydamine inhibits monocyte migration and MAPK activation induced by chemotactic agonists

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1 The present study was aimed to investigate the effect of benzydamine, an anti-inflammatory drug devoid of activity on arachidonic acid metabolism, on monocyte chemotaxis and to define the possible biochemical correlates of activity.

2 Benzydamine inhibited monocyte chemotaxis in response to three classes of chemoattractants: the prototypic CC-chemokine CCL2 (MCP-1), the microbial product fMLP and the complement cascade component C5a. The effect was dose-dependent with IC_{50}'s of 100, 50 and 45 μM for MCP-1/CCL2, fMLP and C5a, respectively. At the dose of 100 μM, the effect resulted in a $50 \pm 10\%$ inhibition of MCP-1/CCL2-induced chemotaxis and 53 ± 6 and $54 \pm 5\%$ inhibitions of chemotaxis in response of fMLP and C5a, respectively ($n = 3$).

3 Receptor expression as well as calcium fluxes in response to chemoattractants were not affected by benzydamine.

4 Benzydamine strongly inhibited chemoattractant-induced activation of the mitogen-activated protein kinase (MAPK) ERK1/2, and of its upstream activator kinase MEK1/2. ERK1/12 activation in response to chemoattractants was 89–98% inhibited by a 100 μM concentration of benzydamine with an IC_{50} of 30 μM.

5 Under the same experimental conditions, pretreatment with 100 μM benzydamine caused a 75–89% inhibition of p38 activation (IC_{50} 25 μM).

6 These results indicate that the anti-inflammatory activity of benzydamine is exerted at multiple levels, including monocyte migration to chemotactic factors associated to a blockage of ERK and p38 MAPK pathways.

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Abbreviations: C5a, complement anaphylatoxin 5a; ERK1/2, extracellular signal-regulated protein kinases 1/2; fMLP, *N*-formyl-L-methionyl-L-leucyl-phenylalanine; MAPK, mitogen-activated protein kinase; MCP-1/CCL2, monocyte chemotactic protein 1; MEK1/2, extracellular signal-regulated kinase kinase 1/2; PMN, polymorphonuclear granulocytes

Introduction

N,*N*-dimethyl-3[(1-benzyl-1*H*-indazol-3-yl)ossi]-1-propanamine (benzydamine) is a nonsteroidal anti-inflammatory drug which is devoid of activity on arachidonic acid metabolism (Cioli *et al*., 1985). Benzydamine is extensively used in clinical practice for the topical treatment of inflammatory conditions, including oral mucositis associated with radiotherapy (Mahon *et al*., 1985; Epstein *et al*., 2001). The anti-inflammatory activity of benzydamine has been recently related to its capacity to inhibit the production of proinflammatory cytokines (TNFα, IL-1β), without significantly affecting other inflammatory cytokines (IL-6, IL-8) and, importantly, anti-inflammatory cytokines (IL-10, IL-1ra) (Sironi *et al*., 1996; 1997; 2000). The mechanisms responsible for the selectivity of benzydamine for pro- versus anti-inflammatory cytokines are a matter of speculation.

Monocytes are members of the human mononuclear phagocyte system, which plays a crucial role in innate immunity against pathogens, and tumor cells, and exerts immunoregulatory functions through cytokine production (Auger & Ross, 1992). Recent studies related the anti-inflammatory activity of benzydamine to its effect on monocytes (Sironi *et al*., 1996; 1997; 2000). In these cells, benzydamine is able to selectively inhibit the production of proinflammatory cytokines.

Leukocyte recruitment is a key event in inflammatory processes, and migration in response to chemotactic agonists is a crucial determinant of leukocyte trafficking. Agonists of different nature function as chemoattractants including microbial products, the prototype of which is *N*-formyl-L-

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methionyl-L-leucyl-phenylalanine (fMLP), chemokines, a superfamily of small (~8–14 kDa) protein, and complement cascade components. Inhibition of chemoattractants and their receptors is a prime target for the development of novel strategies to treat inflammatory disorders (Mantovani & Sozzani, 2000b; Proudfoot, 2002).

The aim of the present study was to extend the investigations on benzydamine activity to chemotaxis, a function shared by immune cells and crucial in inflammation. The results presented here show that benzydamine causes a significant inhibition of the migration of human monocytes, and that this effect likely contributes to its anti-inflammatory activity. Inhibition of the chemotactic response by benzydamine is associated with the inhibition of the ERK and p38 MAPK pathways.

Methods

Reagents

N,*N*-dimethyl-3-[(1-benzyl-1H-indazol-3-yl)ossi]-1-propanamine hydrochloride (benzydamine hydrochloride) was kindly provided by A.C.R.A.F. (S. Palomba-Pomezia, Rome, Italy). fMLP and C5a were from Sigma (St Louis, MO, U.S.A.). MCP-1/CCL2 and IL-8/CXCL8 were from PeproTech Inc. (Rocky Hill, NJ, U.S.A.). Fura-2 acetoxy methyl ester (Fura-2AM; Sigma) was dissolved in acetone, desiccated in aliquots and stored at −20°C in the dark. Just before use, the dye was redissolved in dimethylsulfoxide to a concentration of 1 mM. All the not-specified reagents were from Sigma.

Monocyte purification

Monocytes were obtained from buffy coats of healthy donors through the courtesy of Centro Trasfusionale Ospedale Sacco (Milano, Italy). Monocytes were purified as previously described (Colotta *et al*., 1992). Blood was washed once with saline at 1000 rpm to remove plasma and platelets, and then centrifuged on Ficoll (Biochrom, Berlin, Germany) at 1750 rpm for 30 min at room temperature. PBMC were collected at the interface. Monocytes were then purified by fractionation on a one-step discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient. PBMC obtained from Ficoll gradient were washed twice in saline and suspended in iso-osmotic complete RPMI 1640 medium (Biochrom) adjusted to 285 mOsm with distilled water. The Percoll solution was prepared by mixing 9.25 parts of concentrated Percoll and 0.75 parts ($v\,v^{-1}$) of PBS (Life Technologies, Paisley, U.K.) 10 ×; the osmolarity of this solution was adjusted to 285 mOsm with concentrated PBS (iso-osmotic Percoll solution). Iso-osmotic complete medium and iso-osmotic Percoll solution were mixed to give a 46% Percoll concentration ($v\,v^{-1}$). Cell suspension was carefully layered on the top of the 46% Percoll solution; tubes were spun at 2000 rpm for 30 min at room temperature. Monocytes were recovered at the interface, washed twice in saline and resuspended at 5×10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 1% fetal calf serum (Hyclone, Logan, UT, U.S.A.), and treated as specified below. The purity of cell preparation was ⩾90% as assessed by morphological analysis.

PMN purification

PMNs were purified as previously described (Sozzani *et al*., 1991). After Ficoll gradient PMN layer was recovered from the top of red blood cells pellet, washed twice in saline and suspended in iso-osmotic complete RPMI 1640 medium adjusted to 285 mOsm. Cell suspension was carefully layered on the top of a 63% Percoll solution; tubes were spun at 1500 rpm for 20 min at room temperature. PMNs were recovered at the interface, washed twice in saline and resuspended in RPMI 1640 medium supplemented with 1% fetal calf serum, and treated as specified below. The purity of cell preparation was ⩾95% as assessed by morphological analysis.

Chemotaxis assay

Cells were preincubated with benzydamine (25, 50 or 100 μM) at 37°C for 30 min, and then cell migration was evaluated using a chemotaxis microchamber technique as previously described (Locati *et al*., 2001). Briefly, 27 μl of chemoattractant or control medium (RPMI 1640 with 1% FCS) was added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA, U.S.A.). The cell suspension (50 μl) was seeded in the upper chamber. The two compartments were separated by a PVP polycarbonate filter (5 μm pore size; Neuroprobe). The chamber was incubated at 37°C in humidified atmosphere in the presence of 5% CO_2 for 90 min. In these experimental conditions, using a PBMC suspension (containing 1.5×10^6 monocytes ml^{-1}), only monocytes migrate across the filter and are recovered on its lower side (Falk *et al*., 1980). For PMN chemotaxis, 50 μl of cell suspension (1.5×10^6 PMNs ml^{-1}) was seeded in the upper chamber, PVP-free polycarbonate filters (5 μm pore size; Neuroprobe) were used and the microchamber was incubated at 37°C in humidified atmosphere in the presence of 5% CO_2 for 60 min. Benzydamine was present in both the upper and lower chambers. At the end of incubation, filters were removed, stained and five high-power oil-immersion fields (× 1000) were counted. Under these experimental conditions, the average migrations of monocytes (control cells) to fMLP, MCP-1/CCL2 and C5a were 15, 11 and 10%, respectively, of the input monocyte population. Average migration of PMNs (control cells) was 22% using fMLP as chemoattractant, and 13% using IL-8/CXCL8. Percentages of inhibition were calculated after subtraction of basal migration values.

Measurement of intracellular calcium concentration

Intracellular calcium concentration was monitored using the fluorescent probe Fura-2AM, as previously described (Sozzani *et al*., 1994). Briefly, monocytes (10^7 ml^{-1}) were preincubated with benzydamine (100 μM) at 37°C for 30 min and then, without removing benzydamine, incubated with 1 μM Fura-2AM at 37°C for an additional 30 min. After incubation, the monocytes were washed and resuspended in HBSS (Life Technologies) containing 1.2 mM $CaCl_2$, and kept at room temperature until used. Fura2 fluorescence was measured in a Perkin-Elmer LS 50B spectrophotometer (Perkin-Elmer Instruments, Norwalk, CT, U.S.A.) at 37°C, with cells (5×10^6 ml^{-1}) continuously stirred. Samples were excited at

340 and 380 nm, and emission at 487 nm was continuously recorded.

FACS analsyis

Cell staining was performed with a biotinylated mouse anti-h-CCR2 antibody (R&D, Minneapolis, MN, U.S.A.), followed by incubation with streptavidin-phycoerythrin (Pharmingen, San Diego, CA, U.S.A.). Analysis of fluorescence was performed by a FACStar Plus (Becton Dickinson, Mountain View, CA, U.S.A.) calibrated with Calibrite beads (Becton Dickinson).

Cell stimulation and Western blotting

Monocytes (5×10^6 cells per sample) were preincubated in the presence or absence of benzydamine at a concentration of 100 μM (unless otherwise specified) at 37°C for 30 min, and then stimulated with chemotactic agonists for 3 min. In the range of concentration tested (up to 100 μM), benzydamine did not affect cell viability, as assessed by Trypan Blue dye exclusion assay. Chemoattractant optimal working concentrations were: C5a, 100 ng ml^{-1}; fMLP, 100 nM; MCP-1/CCL2, 100 ng ml^{-1}. Stimulation was terminated by adding 10 ml of ice-cold PBS/1 mM Na_3VO_4. Cells were pelletted and lysed with 100 μl of lysis buffer (20 mM Tris-HCl pH 7.4; 0.2 mM Na_3VO_4; 2 mM EDTA; 5 mM β-mercaptoethanol; 25 mM NaF; 25 mM sodium pyrophosphate; 25 mM disodium β-glycerophosphate; 1% Triton X-100; 2000 U ml^{-1} aprotinin; 1 mM PMSF; 0.1 mM leupeptin; 0.05 mM pepstatin). Protein concentration was evaluated according to Bradford's method.

Proteins (100 μg) were separated on 10% SDS–PAGE and transferred to nitrocellulose paper (Bond-NC; Whatman, Maldstone, U.K.). Nonspecific binding sites were blocked by incubating the membrane in 5% non-fat dry milk/Tris-buffered saline Tween (TBS-T = 20 mM Tris pH 7.2; 150 mM NaCl; 0.1% Tween 20) for 1 h at room temperature. The membrane was then incubated (overnight at 4°C) with antibodies that specifically detect the phosphorylated (i.e. activated) form of ERK1/2, p38 and MEK1/2 (Cell Signaling Technology, Beverly, MA, U.S.A.) at the indicated dilution. Next, it was washed with TBS-T and incubated for 1 h at room temperature with the HRP anti-rabbit antibody (Amersham, Buckinghamshire, U.K.) at 1:1000 dilution. Finally, it was washed with TBS-T and immunoreactivity was detected by ECL (Amersham). Results were analyzed by densitometric analysis.

Statistical analysis

One-way ANOVA with Dunnet's post-test was used for statistical analysis ($P<0.05$).

Results

Benzydamine inhibits chemotaxis in human monocytes

In a first series of experiments, we examined whether benzydamine could affect the responsiveness of human monocytes to agonists representative of different classes of chemotactic agents. Monocytes were preincubated with different concentrations of benzydamine and tested for migration in response to fMLP (microbial product), MCP-1/CCL2 (chemokine) or C5a (complement cascade component). Benzydamine-treated monocytes showed a reduced chemotaxis when compared to untreated cells. As shown in Figure 1a, benzydamine caused a significant ($P<0.05$) inhibition of the migration of human monocytes to chemoattractants. In these experimental conditions, 50% of inhibition was reached at benzydamine concentrations of 100, 50 and 45 μM for MCP-1/CCL2, fMLP and C5a, respectively ($n=3$). Benzydamine concentrations active on monocyte chemotaxis (25–100 μM = 7.8–31.2 μg ml^{-1}) are similar to those shown to inhibit cytokine production and fall in the therapeutic range of concentration observed *in vivo* (Schoenwald *et al*., 1987; Sironi *et al*., 1996).

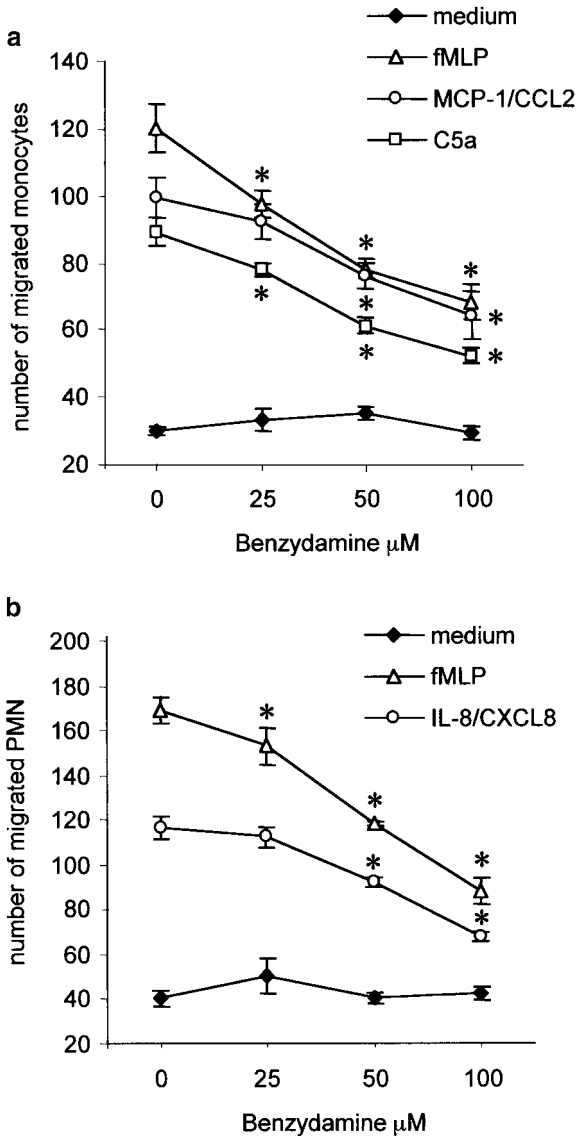

Figure 1 Effect of benzydamine on chemotaxis: Freshly isolated human PBMC (panel (a)) or PMNs (panel (b)) were preincubated at 37°C with different benzydamine concentrations for 30 min, before being tested for their ability to migrate toward MCP-1/CCL2 (50 ng ml^{-1}), C5a (50 ng ml^{-1}), fMLP (10^{-7} M) or IL-8/CXCL8 (100 ng ml^{-1}). Data reported are means ± s.e.m. of three independent experiments, each one performed in triplicate (*$P<0.05$).

The reduced chemotactic response could derive from a decrease in the expression of surface chemoattractant receptors. To exclude this possibility, monocytes were treated with benzydamine and the expression of CCR2 was subsequently evaluated. CCR2 is the only MCP-1/CCL2 receptor identified so far, and it is a major chemokine receptor involved in monocyte migration (Murphy *et al*., 2000). No difference was observed in CCR2 expression on monocytes incubated with benzydamine in comparison with untreated monocytes (Figure 2). Similarly, benzydamine did not modify cytosolic $[Ca^{2+}]_i$ increase in response to fMLP or MCP-1/CCL2 (Figure 3).

The effect of benzydamine on PMNs, another major subset of leukocytes involved in inflammation, was also investigated. In these experiments, the chemoattractants used were fMLP and IL-8/CXCL8. Similar to what was observed for monocytes, benzydamine pretreatment inhibited PMN chemotaxis, resulting in 50% of inhibition at 70 and 77 μM benzydamine for fMLP and IL-/CXCL8, respectively (Figure 1b; $n=3$, $P<0.05$). These results indicate that benzydamine acts in a similar manner on multiple inflammatory leukocyte populations. Since the effect of benzydamine on monocyte functions has been the object of extensive investigations (Sironi *et al*., 1996; 1997; 2000), subsequent studies focussed on this cell population.

Benzydamine inhibits MAPK activation in response to chemotactic agonists

Chemoattractant receptors activate the ERK and p38 MAPK pathways (Bokoch, 1995; Wenzel-Seifert & Seifert, 2001). MAPK activation is implicated both in chemotaxis and in the synthesis of proinflammatory cytokines; therefore, it was of interest to evaluate the effect of benzydamine on MAPK activation. Human monocytes were pretreated with benzydamine and then stimulated with fMLP, C5a or MCP-1/CCL2. Activation of MAPK occurs through phosphorylation of both a threonine and a tyrosine residue in a Thr-Xaa-Tyr motif close to the active site. The activation of ERK and p38 was studied by Western blot analysis using antibodies that specifically detect the dually phosphorylated form of MAPK.

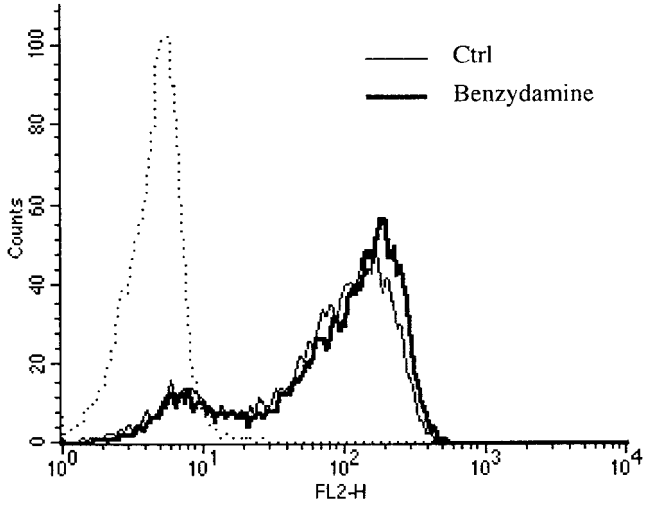

Figure 2 Effect of benzydamine on CCR2 expression: Flow cytometry analysis of CCR2 in monocytes incubated in the absence or presence of 100 μM benzydamine for 2 h. Dotted line represents the reactivity of the isotype-matched control monoclonal antibody. Results of a single experiment, representative of at least three, are shown.

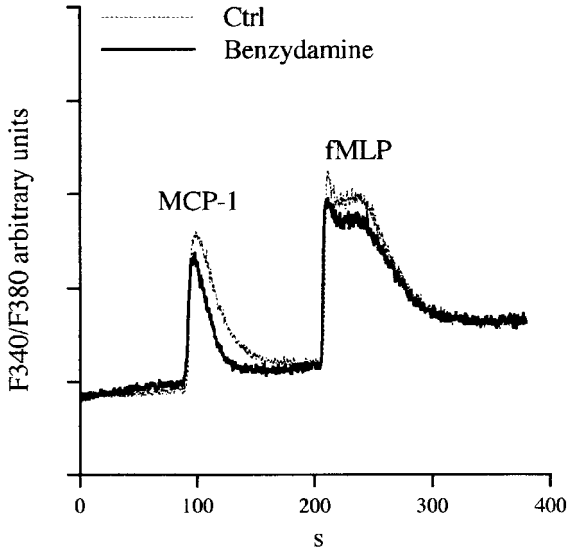

Figure 3 Effect of benzydamine on chemoattractant-induced calcium fluxes: Freshly isolated human monocytes were preincubated in the absence or presence of 100 μM benzydamine for 30 min at 37°C, incubated with Fura-2AM (1 μM) for 30 min at 37°C still in the presence of the drug, washed, and then exposed in cuvettes (10×10^6 ml^{-1}) to MCP-1/CCL2 (100 ng ml^{-1}) or fMLP (10^{-7} M). One experiment representative of two is shown. Results are expressed as ratio of fluorescence at two excitation wavelengths (340 and 380 nm).

In a first series of experiments, the effect of benzydamine on ERK pathway was investigated. Figure 4 shows that ERK1/2 activation in response to all the chemotactic agents tested was strongly inhibited by benzydamine. Figure 4a shows that the effect of benzydamine on fMLP ERK1/2 activation was concentration-dependent, being already detectable at 25 μM and maximal at 100 μM of the drug ($IC_{50}=30$ μM). At the optimal concentration of 100 μM, the drug similarly inhibited the effects of the other chemotactic agonists tested (88.0±10.1% for MCP-1/CCL2; 88.5±1.9% for C5a; 98±1.5% for fMLP; $n=5$) (Figure 4, panels b and c). Activation of ERK1/2 is achieved through specific upstream kinases, namely MEK1/2 (Widmann *et al*., 1999). The specific ERK cascade inhibitor PD98059 acts as an inhibitor of MEK kinases (Yen *et al*., 1997). Therefore, benzydamine activity on ERK activators was evaluated. Similar to the results obtained with ERK1/2, benzydamine was able to suppress MEK1/2 activation induced by chemokines and classical chemoattractants (Figure 5, panels a and b) with percentages of inhibition of 91.9±5.2% (MCP1/CCL2), 98±0.9% (fMLP) or 98.5±0.4% (C5a) ($n=5$).

The study was then extended to the other major MAPK pathway, the p38 kinase cascade. Similar to what was observed for ERK pathway, benzydamine inhibited p38 activation in chemoattractant-stimulated cells. The effect of benzydamine on fMLP-induced p38 activation was concentration-dependent, with an IC_{50} of 25 μM, and maximal effect was detected at 100 μM with an inhibition of 89.0±8.6% (Figure 6, panel a). At this concentration, p38 activation by C5a and MCP-1/CCL2 was inhibited to a similar extent (75.1±19.9 and 75.5±11.1%, respectively; $n=5$) (Figure 6, panels b and c).

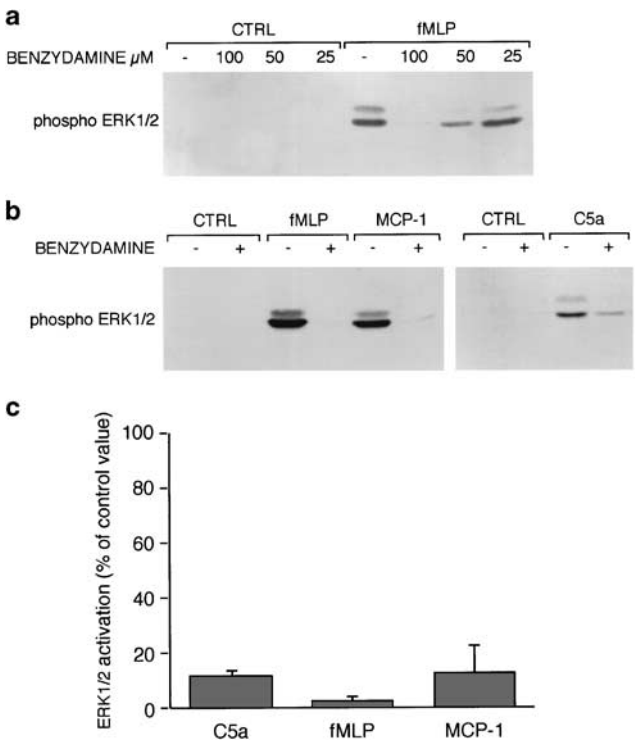

Figure 4 Effect of benzydamine on ERK1/2 activation: Freshly isolated human monocytes were preincubated with different concentrations of benzydamine (panel (a)) or with 100 μM benzydamine (panels (b, c)) at 37°C for 30 min, and then stimulated with chemotactic agonists for 3 min. Cells were lysed, subjected to SDS–PAGE, and blotted with an antiactive ERK1/2 antibody. Panel (a): dose–response analysis of benzydamine effect on fMLP-stimulated ERK1/2 activation. The representative immunoblot is one of two independent experiments with similar results. Panel (b): one representative immunoblot of five experiments. Panel (c): densitometric analysis of immunoblots. Data reported are means ± s.e.m. of five experiments.

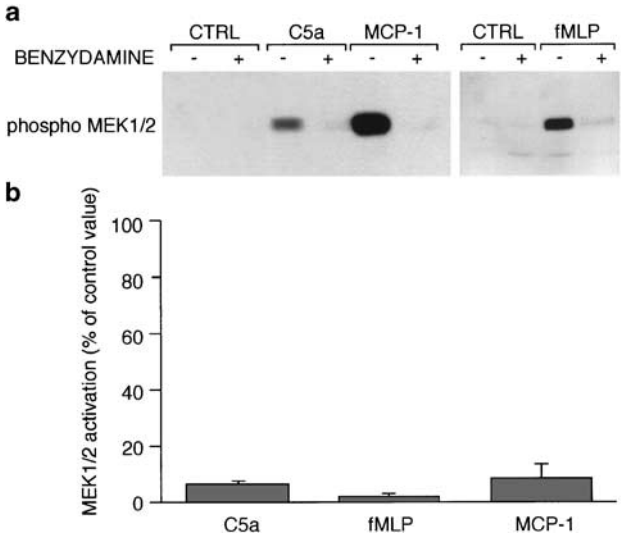

Figure 5 Effect of benzydamine on MEK1/2 activation: Freshly isolated human monocytes were preincubated with 100 μM of benzydamine at 37°C for 30 min, and then stimulated with chemotactic agonists for 3 min. Cells were lysed, subjected to SDS–PAGE, and blotted with an antiactive MEK1/2 antibody. Panel (a): one representative immunoblot of five experiments. Panel (b): densitometric analysis of immunoblots. Data reported are means ± s.e.m. of five experiments.

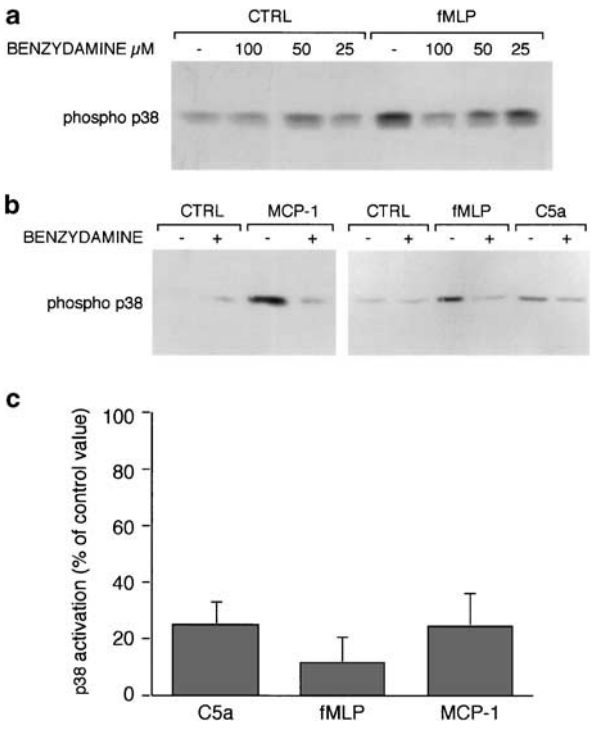

Figure 6 Effect of benzydamine on p38 activation: Freshly isolated human monocytes were preincubated with different concentrations of benzydamine (panel (a)), or with 100 μM benzydamine (panels (b, c)) at 37°C for 30 min, and then stimulated with chemotactic agonists for 3 min. Cells were lysed, subjected to SDS–PAGE, and blotted with an antiactive p38 antibody. Panel (a): dose–response analysis of benzydamine effect on fMLP-stimulated p38 activation. The representative immunoblot is one of two independent experiments with similar results. Panel (b): one representative immunoblot of five experiments. Panel (c): densitometric analysis of immunoblots. Data reported are means ± s.e.m. of five experiments.

Discussion

Benzydamine is a nonsteroidal anti-inflammatory drug widely used for the topical treatment of inflammatory conditions. Benzydamine has no effect on arachidonate metabolism and its molecular target is unknown (Sironi *et al*., 2000). The data presented here extend our knowledge on the mechanism of action of this drug, and show that benzydamine is able to limit the recruitment of leukocytes to the site of inflammation by inhibiting cell migration in response to chemotactic signals. This effect is not selective for monocytes, but a similar inhibition was observed with PMNs. Reduced chemotactic response was not due to reduced chemotactic receptor expression, nor associated with a decrease of chemoattractant-induced calcium fluxes. Conversely, inhibition of chemotaxis by benzydamine was apparently related with inhibition of MAPK activation, a pathway that was implicated in cell migration (Bokoch, 1995).

Mitogen-activated protein kinases (MAPKs) are enzymes expressed in all eukaryotic cells, and transduce extracellular signals into cellular responses. They play important roles in cell proliferation, apoptosis, differentiation, cell migration and cytoskeleton remodeling (English *et al*., 1999; Garrington & Johnson, 1999; Widmann *et al*., 1999). The mammalian MAPK family includes at least four members: ERKs, p38 MAPKs, JNKs and BMKs (ERK5). MAPK cascades consist

of a module that includes three kinases. These establish a sequential activation pathway comprising a MAPK kinase kinase (MKKK), a MAPK kinase (MKK) and MAPK. Many different receptor types are able to activate MAPK cascades by multiple mechanisms including tyrosine kinases receptors, G-protein-coupled receptors and cytokine receptors (English *et al*., 1999; Widmann *et al*., 1999).

MAPK were among the first kinases to be implicated in the synthesis of proinflammatory cytokines and several inhibitors of cytokine production exert their activity by blocking MAPK activation (Mantovani *et al*., 2000a). In the late 1980s, a new class of anti-inflammatory agents named 'cytokine suppressive anti-inflammatory drug' (CSAID™) has been developed. The prototypic compound of this class is SB203580, a drug that showed a novel mechanism of action (Lee *et al*., 1988; Lee *et al*., 1993; Badger *et al*., 1996). These pyridinyl imidazole compounds have potent inhibitory effects on cytokine production *in vitro*, and *in vivo* they can attenuate the inflammatory component of diseases in the absence of generalized immunosuppression. The molecular targets of CSAID™ were identified as a pair of closely related mitogen-activated protein kinase homologues alternatively termed 'cytokine-suppressive binding protein' (CSBP) (Lee *et al*., 1994; 1996), p38 (Han *et al*., 1994) or RK (Rouse *et al*., 1994). Inhibitors of p38 MAPK were shown to suppress the synthesis of pro- and anti-inflammatory cytokines in a nonselective manner (Lee *et al*., 1994). In contrast, ERK1/2 inhibitors block TNFα and IL-1β (the two main proinflammatory cytokines), but not IL-10 (an anti-inflammatory cytokine) (Foey *et al*., 1998). MAPK inhibitors have been shown to possess significant therapeutic benefits in a number of models of inflammation, including endotoxin shock (Badger *et al*., 1996; Nick *et al*., 2000), collagen-induced arthritis (Badger *et al*., 1996), pulmonary inflammation (Nick *et al*., 2000) and gastritis (Takahashi *et al*., 2001). Activation of MAPK is necessary for chemotaxis, and previous studies reported that MAPK inhibitors decrease cell migration in response to chemoattractants (Rousseau *et al*., 1997; Yen *et al*., 1997; Boehme *et al*., 1999; Matsumoto *et al*., 1999; Ayala *et al*., 2000). Although the chemotaxis process is the result of multiple signalling pathways (Wenzel-Seifert & Seifert, 2001), it is likely that blocking of the MAPK pathway contributes to the inhibition of monocyte chemotaxis by benzydamine.

Benzydamine is an inhibitor of cytokine (TNFα, IL-1β) production (Sironi *et al*., 1996; 1997; 2000). Since MAPK inhibitors block cytokine production, it could be postulated that benzydamine acts by blocking this pathway. Indeed, we report that benzydamine strongly inhibits both ERK1/2 and p38 MAPK activation induced by chemotactic factors. However, MAPK inhibition is selective for chemoattractant-stimulated MAPK activation, since benzydamine does not affect the lipopolysaccharide (LPS)-stimulated activation (data not shown). Chemoattractants and LPS can activate MAPK by different mechanisms (Bokoch, 1995; Clapham & Neer, 1997; English *et al*., 1999; Widmann *et al*., 1999; Akira *et al*., 2001; Wenzel-Seifert & Seifert, 2001); hence, it is not surprising that benzydamine exerts its inhibitory activity on chemoattractant signalling without affecting the LPS functions, acting upstream of the MKKK–MKK–MAPK module.

Benzydamine IC_{50}'s for monocyte migration were slightly higher than those for MAPK activation (e.g. 50 μM and about 30 μM for fMLP-induced chemotaxis and MAPK, respectively). Therefore, MAPK pathway is apparently slightly more sensitive than chemotaxis to the effect of this drug. In parallel experiments, PD98059, a well-known inhibitor of ERK1/2, inhibited chemotaxis and ERK1/2 with respective IC_{50}'s of 35 and 5 μM (data not shown). Inhibition of chemotaxis and p38 activation by SB203580 (an inhibitor of p38) showed IC_{50}'s of 16 and 2 μM, respectively (data not shown). These results confirm the previous observation showing that both ERK1/2 and p38 are involved in cell migration, although the concentrations required to inhibit chemotaxis are somewhat higher than those necessary to inhibit enzyme activation (Rousseau *et al*., 1997; Yen *et al*., 1997; Boehme *et al*., 1999; Matsumoto *et al*., 1999; Ayala *et al*., 2000). The exact reason for this difference is not known. However, it must be considered that these two biological responses are tested using different technical conditions. The evaluation of the MAPK pathway is performed after a few minutes of stimulation (1–5 min), while cell migration is tested following a long exposure (90 min) of monocytes to a gradient of chemotactic agonist. All in all, these results indicate that inhibition of the MAPK pathway by benzydamine is likely to represent a key step in the mechanism of action of this drug on monocyte migration. Although additional effect(s) of benzydamine on other activated signalling events cannot be excluded on the basis of this study, it must be noted that these *in vitro* results are observed in the range of the therapeutic concentrations obtained *in vivo* (Schoenwald *et al*., 1987; Sironi *et al*., 1996).

In summary, the results presented in this study show that benzydamine inhibits the migration of inflammatory leukocytes, and that this effect is associated with inhibition of the MAPK pathway. Inhibition of MAPK activation and cell migration in response to chemotactic agents is likely to contribute to the anti-inflammatory activity of this compound.

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