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β_2-agonist-induced inhibition of neutrophil chemotaxis is not associated with modification of LFA-1 and Mac-1 expression or with impairment of polymorphonuclear leukocyte antibacterial activity

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Patients with chronic obstructive lung disorders often show increased susceptibility to airway infections. As β_2-adrenoceptor agonists, in addition to reversing the contractile response of bronchial smooth muscles, may inhibit a variety of inflammatory and immuno-effector cell functions, it is possible that these drugs interfere with host defence mechanisms.

The present study was designed to test *in vitro* whether fenoterol, a short-acting β_2-adrenoceptor agonist, could modify human blood neutrophil recruitment and antimicrobial activity.

Pre-exposure to fenoterol significantly reduced neutrophil migration towards the complement component C5a, at concentrations ranging from 10^{-7} M to 10^{-5} M, or towards lipopolysaccharide, at a concentration of 10^{-5} M ($P<0{\cdot}05$, each comparison). In contrast, the drug (10^{-8}–10^{-5} M) did not significantly modify the increased expression of lymphocyte function-associated antigen (LFA-1, i.e. CD11a/CD18) the macrophage antigen-1 (Mac-1, i.e. CD11b/CD18) induced by *N*-formylmethionylleucylphenylalanine (fMLP) ($P>0{\cdot}05$, each comparison). Finally, incubation of neutrophils with fenoterol (10^{-8}–10^{-5} M) did not significantly influence phagocytosis or intracellular killing of bacteria (*Staphylococcus aureus*) or H_2O_2 release induced by tetradecanoyl-phorbol-acetate ($P>0{\cdot}1$ for each comparison).

These results suggest that short-acting β_2-adrenoceptor agonists, such as fenoterol, are able partially to reduce neutrophil recruitment in the airways without interfering with the processes involved in phagocytic activity against bacteria.

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Introduction

The progressive tissue damage which characterizes chronic bronchitis is related, at least in part, to the presence of excessive numbers of activated polymorphonuclear leukocytes, such as neutrophils (1,2). Indeed, in addition to their phagocytic activity, neutrophils also act as secretory cells able to release preformed proteins stored in the cytoplasmic granules as well as toxic oxygen radicals (3–5). The granule-associated preformed proteins, including cathepsin G, elastase, proteinase-3, collagenase and gelatinase, damage bronchial and lung connective tissue components (6–8), while oxygen radicals, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), hypochlorous acid ($HClO^-$), are highly toxic for ciliated epithelial cells (4,5).

In addition to their potential role in damaging human tissues, neutrophils can also protect the host against infection (9). The usual steps resulting in pathogen clearance are neutrophil recruitment into the involved tissues, phagocytosis of invading micro-organisms, release of proteinases and generation of oxygen metabolites (4,9). However, when neutrophil recruitment and activation is continuous or excessive, dysfunctions of the host defence mechanisms can be hypothesized while the exaggerated inflammatory response may contribute to tissue damage (3,10). This phenomenon has been described in subjects with cystic fibrosis and with bronchiectasis (11,12) and recently demonstrated in chronic bronchitis patients colonized by *Streptococcus pneumoniae* (13).

Neutrophil dysfunction may be induced or enhanced by drugs administered to patients with chronic inflammatory diseases of the airways. Drugs able to interfere with neutrophil functions include corticosteroids (14,15), antibiotics (16) and β_2-adrenoceptor agonists, which are often prescribed to treat reversible airway obstruction in chronic

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airway diseases (17). Indeed, it has been demonstrated that β_2-adrenoceptor agonists are able to modulate the functions of a variety of cells, including phagocytes (17–20).

The present study was designed to evaluate whether fenoterol, a short-acting β_2-adrenoceptor agonist, interferes with neutrophil functions and therefore facilitates the recurrence of respiratory infections associated with chronic airway diseases.

Using blood neutrophils isolated from healthy volunteers, we tested the *in vitro* the effect of fenoterol on: 1. the cell chemotaxis; 2. the expression of adhesion molecules [lymphocyte function-associated antigen (LFA-1 or CD11a/CD18) and macrophage antigen-1 (Mac-1 or CD11b/CD18)]; 3. the antimicrobial activity against bacteria (*Staphylococcus aureus*); and 4. respiratory burst induced by tetradecanoylphorbolacetate.

Materials and Methods

STUDY POPULATION

Twenty-five healthy volunteers (14 men and 11 women aged 18–27 years) entered the study. Exclusion criteria were history of respiratory infection in the previous 4 weeks and intake of drugs known to affect biological function of neutrophils and/or mononuclear cells. The protocol was approved by the Giannina Gaslini Institute Ethical Committee and all subjects, after being informed of the nature and aim of the study, gave written consent.

NEUTROPHIL PURIFICATION

The isolation of neutrophils from peripheral blood was performed by discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) (21). Briefly, 10 ml of 6% dextran (PM 70 000, SIFRA SpA, VR, Italy) in 0.9% NaCl were added to an equal volume of heparinized blood, mixed gently and incubated at 37°C. After 30–40 min, the upper phase was collected, washed once in PBS, resuspended in 1·5 ml of 1·070 g ml^{-1} Percoll with 5% FCS (Flow ICN, Irvine, Scotland, U.K.) and layered on discontinuous Percoll gradients with the following volumes and densities (g ml^{-1}): 1·5 ml 1·100, 3 ml 1·090, 3 ml 1·085 and 3 ml 1·080. The Percoll gradient densities were obtained by mixing nine parts of Percoll with one part of 10 × HBSS (90% Percoll) and then diluting the 90% Percoll solution with 1 × HBSS containing 5% FCS. The neutrophils recovered between 1085 and 1080 g/ml^{-1} of Percoll were washed twice in PBS and resuspended at 10^6 cells ml^{-1} in complete medium. Neutrophils recovered were 95% pure (the remaining 5% were mononuclear cells) as established by Diff-Quick staining (Merz+Dade, Dudingen, Switzerland), and 99% viable, as determined by the trypan blue dye exclusion test (Flow ICN).

NEUTROPHIL CHEMOTAXIS ASSAY

Neutrophil chemotaxis was tested using 48-well Boyden microchambers (Neuro Probe Inc., Cabin John, MD, U.S.A.). Fenoterol, kindly provided as pure powder by Boheringer Ingelheim, Italy, was resuspended in distilled water to prepare 1 mM stock solution. To evaluate the effect of fenoterol on neutrophil chemotaxis, cells were preincubated for 3 h with different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) before performing the assay. Preliminary experiments demonstrated that preincubation of neutrophils with fenoterol for 3 h did not induce desensitization, as also shown for airway epithelial cells (22). The lower wells of the Boyden microchambers were loaded with two different stimuli: 1. C5a (active fragment of complement cascade) (0·1 mg ml^{-1}, Sigma Chemical Co., St. Louis, MO, U.S.A.) or 2. lipopolysaccharide (LPS, 0·2 μg ml^{-1}, Sigma Chemical Co.) (23). The lower wells were then covered with a polycarbonate membrane with 3 μm pores (Costar Corp., Cambridge, MA, U.S.A.) and 50 μl aliquots of cell suspensions (10^6 cells ml^{-1}) were pipetted into the upper wells of the chemotaxis chamber. After 30 min incubation at 37°C in 5% CO_2, the membrane was detached and the neutrophils, which had migrated onto the lower side of the membrane, were fixed, stained with Diff-Quick and counted under a light microscope. All the experimental conditions were tested in triplicate and the data expressed as number of cells migrated in 10 high power fields (HPF, magnification × 400).

Controls were performed by incubating neutrophils with complete medium for 3 h prior to performing the assay.

EXPRESSION OF LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN (LFA-1)

Neutrophils (2×10^6 cells ml^{-1}) were seeded into 96 round-bottom well-microtiter plates (Costar Corp.) and pre-exposed to different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) for 3 h. Cells were then stimulated with *N*-formylmethionylleucylphenylalanine (fMLP) (Sigma Chemical Co.) 10^{-6} M for 1 h. After incubation, the plate was centrifuged and the cells washed and resuspended in 100 μl of the staining medium with 1% fetal calf serum (Biofluids) and 0·2% sodium azide (Sigma Chemical Co.) in PBS (Flow ICN). The cells were stained with 2·5 μl of PE-conjugated monoclonal antibody anti-CD11a (recognizing the α1 chain specific to LFA-1) (Serotec, Oxford, U.K.) and incubated for 30 min at 4°C. The cells were then washed twice and analysed by flow cytometry using LYSYS II software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) (24). Data are expressed as mean fluorescence channel (mfc).

EXPRESSION OF MACROPHAGE ANTIGEN-1 (MAC-1)

Neutrophils (2×10^6 cells ml^{-1}) were seeded into 96 round-bottom well-microtiter plates and pre-exposed to different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) for 3 h. Cells were then stimulated with fMLP 10^{-6} M for 5 min. After incubation, the plate was centrifuged and the cells washed, resuspended and stained with 2·5 μl of

PE-conjugated monoclonal antibody anti-CD11b (Serotec, Oxford, U.K.) and incubated as described above.

BACTERIAL PREPARATION

Phagocytosis and intracellular killing of bacteria by neutrophils were measured as previously described (25). Briefly, an overnight culture of *S. aureus* strain ATCC 25923 (Rockville, MD, U.S.A.), kept in 5 ml of BHI broth (Difco Laboratories, Detroit, Michigan, U.S.A.), was pelleted ($1300 \times g$), resuspended in 5 ml of deionized water and labelled with 3 μg ml^{-1} of 4′, 6′-diamidine-2 phenylindole 2 HCl (DAPI) (Sigma Chemical Co.), a fluorochrome binding to the A+T-rich regions of double-stranded DNA. After 10 min incubation at room temperature, bacteria were washed and resuspended in complete medium without antibiotics and with 50% human autologous serum at a concentration of 10^8 colony-forming units ml^{-1} at 650 nm. Bacteria were incubated for 30 min at 37°C in 5% CO_2, to promote opsonization, and then washed. Propidium iodide (PI) (Sigma Chemical Co.), a viability dye excluded by the cytoplasmic membrane of live cells but able to bind to DNA of dead bacteria, was added at a final concentration of 2 mg ml^{-1} to the bacterial suspension (DAPI-PI stained bacteria).

NEUTROPHIL PHAGOCYTOSIS AND INTRACELLULAR KILLING OF BACTERIA

Neutrophils, preincubated with different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) for 3 h, were added to the DAPI-PI stained bacteria suspension at a final bacteria/phagocyte ratio of 30:1 (25). After 1 h incubation, 60 ml aliquots of the neutrophil/bacteria suspensions were collected from the tubes and, mixed with 8 μl of 3 μM acridine orange (Sigma Chemical Co.), to enhance intracellular details on fluorescence microscopy examination. Cytocentrifuge preparations were set, dry-fixed with cyanoacrylate and evaluated by epi-illumination UV microscopy using two different filters for DAPI (excitation 365 nm, emission >420 nm) and PI (excitation 546 nm, emission >590 nm). With this staining, viable bacteria appear blue, while dead bacteria appear red. For each experimental condition, a minimum of 300 neutrophils was evaluated.

HYDROGEN PEROXIDE PRODUCTION BY ACTIVATED NEUTROPHILS

In order to evaluate the possible effects of fenoterol on the respiratory burst of neutrophils, hydrogen peroxide (H_2O_2) production was measured using a colorimetric method, based on the absorption of red ferrothiocyanate in the presence of hydrogen peroxide (26). H_2O_2 production was evaluated in neutrophils (2×10^6 cells ml^{-1} in Krebs–Ringer phosphate buffer) preincubated with different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) for 3 h and stimulated for 1 h with a cell membrane activator, tetradecanoyl-phorbol-acetate (TPA, 100 ng ml^{-1}) (Sigma Chemical Co.). NaN_3 2 mM (Sigma Chemical Co.) was added to each experimental condition to inhibit H_2O_2 breakdown. After incubation, proteins were precipitated on ice with trichloroacetic acid (10% w/v, final concentration) (Carlo Erba Reagents, Milan, Italy) and centrifuged at $1000 \times g$. Then, 0·4 ml of ferrous ammonium sulphate

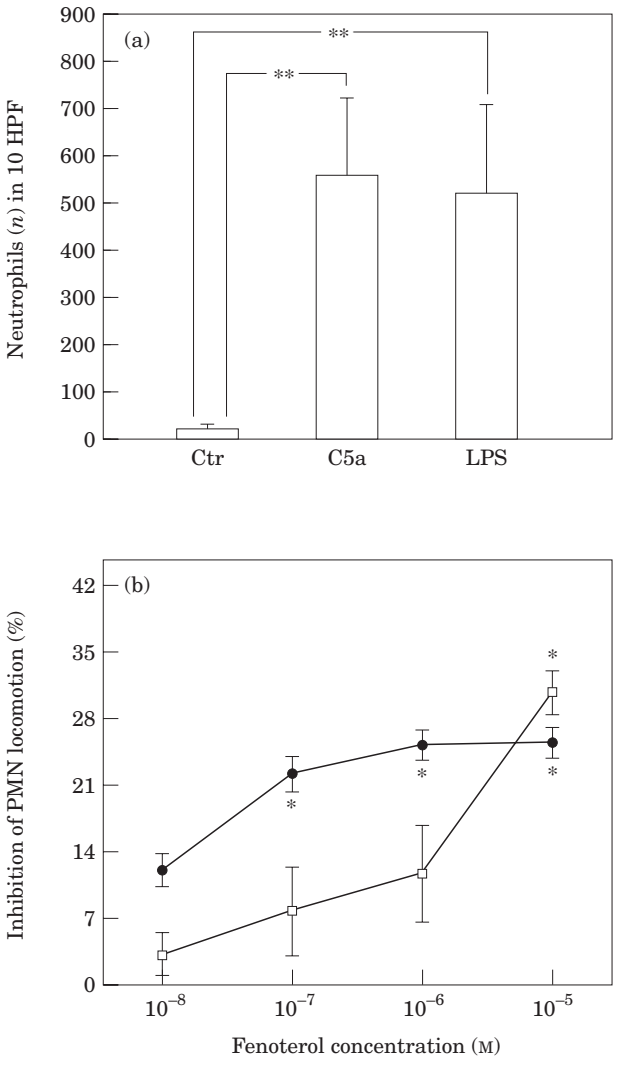
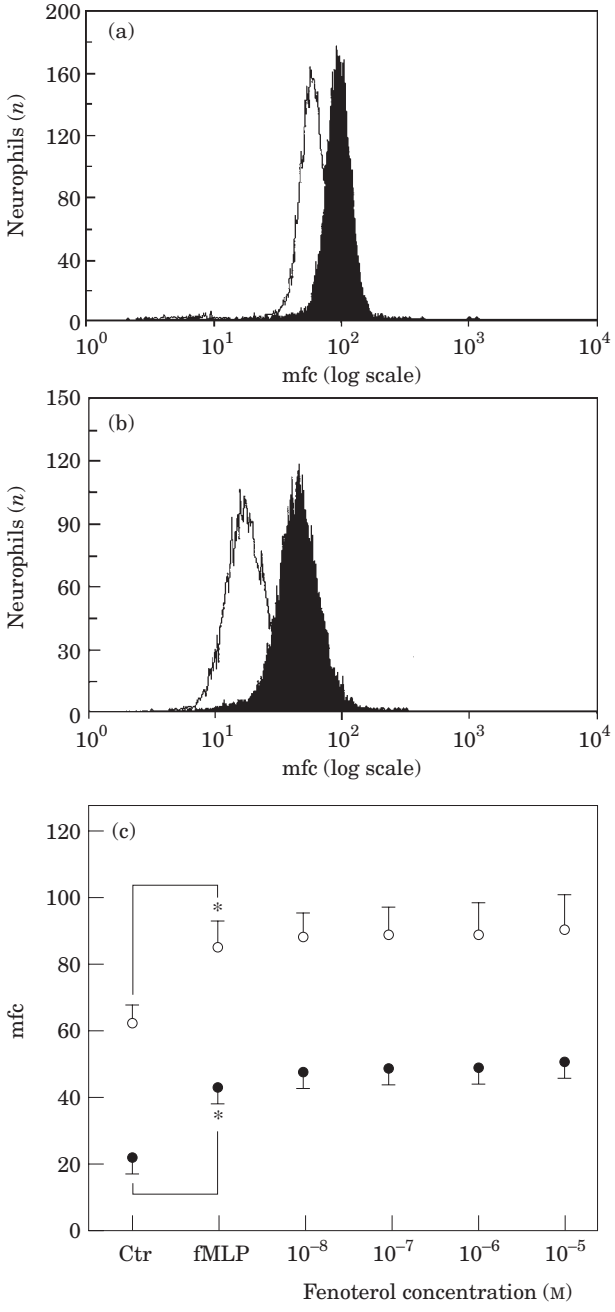


FIG. 1. Neutrophil locomotion toward C5a (0·1 μg ml^{-1}) or lipopolysaccharide (LPS, 0·2 μg ml^{-1}) and inhibition of neutrophil chemotaxis in the presence of fenoterol. (a) Neutrophil locomotion is expressed on the ordinate as the number of cells counted in 10 high power fields (HPF), while the different experimental conditions are shown on the abscissa. (b) The chemotaxis of neutrophils pretreated with fenoterol toward C5a (●) or toward LPS (□) is expressed as a percentage of inhibition on the ordinate, while the different concentrations of fenoterol (from 10^{-8} to 10^{-5} M) are shown on the abscissa. In (a), the data, expressed as mean ± s.e.m., represent the results of 20 experiments, while in (b), each point represents the mean of 10 experiments. ** = P=0·001 *vs.* control; * = P<0·05 *vs.* control.

10 mM and 0·2 ml of potassium thiocyanate 2·5 M (Carlo Erba Reagents) were added to the cell supernatants. The absorbance of red ferrothiocyanate complexes was finally measured spectrophotometrically at 480 nm wavelength and the values, plotted with a standard calibration curve, were expressed as H_2O_2 nmol 10^{-6} cells.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (s.e.m.). Statistical comparisons between different culture conditions were made using the Student's *t*-test. Data were considered significant when the *P*-value was <0·05.



Results

NEUTROPHIL LOCOMOTION

Both C5a and LPS were able significantly to increase neutrophil locomotion [random migration (ctr) = 20·4 ± 4·6 neutrophils $10HPF^{-1}$, C5a = 553·2 ± 164·7 neutrophils $10HPF^{-1}$, LPS = 516·0 ± 201·2 neutrophils $10HPF^{-1}$, $P<0·001$ for each comparison *vs* ctr] [Fig. 1(a)]. The number of neutrophils migrating towards C5a was significantly reduced in the presence of fenoterol concentrations from 10^{-7} to 10^{-5} M ($P>0·05$ for each comparison) [Fig. 1(b)]. A similar effect of the drug was also observed in neutrophil chemotaxis towards LPS, even if, under these experimental conditions, statistical significance was reached only at the highest concentration, i.e. 10^{-5} M ($P<0·05$) [Fig. 1(b)]. Cell viability, as assessed by the trypan blue exclusion test, was not altered by any concentration of fenoterol ($P>0·05$) (not shown).

LFA-1 AND MAC-1 ADHESION MOLECULE EXPRESSION

Stimulation of neutrophils with fMLP induced a statistically significant upregulation of LFA-1 expression being 61·35 ± 1·71 mfc in unstimulated cultures and 87·74 ± 5·14 mfc in fMLP-stimulated cultures ($P<0·01$, comparison with control), [Fig. 2(a)]. Similar results were obtained when Mac-1 expression was evaluated ($P<0·01$, comparison with control) [Fig. 2(b)]. The presence of fenoterol did not modify the expression of LFA-1 and of Mac-1 on stimulated neutrophils [Fig. 2(c)].

FIG. 2. CD11a and CD11b expression on neutrophils stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). (a) Flow-cytometric histogram of CD11a expression on neutrophils. The neutrophil number is shown on the ordinate, while the red fluorescence intensity given by anti-human CD11a PE-conjugated monoclonal antibody is shown on the abscissa as mean fluorescence channel (mfc) of each cell. The peak on the left represents unstimulated cells (Basal) and the peak on the right indicates neutrophils stimulated with fMLP. (b) Flow-cytometric histogram of CD11b expression on neutrophils. The neutrophil number is shown on the ordinate, while the red fluorescence intensity given by anti-human CD11b PE-conjugated monoclonal antibody is shown on the abscissa as mfc of each cell. (c) Effect of fenoterol on CD11a (○) or CD11b (●) expressions on neutrophils stimulated by fMLP. The fluorescence intensity given by anti-human CD11a or anti-human CD11b PE-conjugated monoclonal antibody is shown on the ordinate as the mfc of each cell, while the different experimental conditions are expressed on the abscissa. The data, expressed as mean ± s.e.m., represent the results of 18 experiments. * = $P<0·01$ *vs*. control.

NEUTROPHIL PHAGOCYTOSIS AND INTRACELLULAR KILLING OF *STAPHYLOCOCCUS AUREUS*

After 1 h incubation, high proportions (89·7 ± 2·8%) of neutrophils had detectable bacteria in their cytoplasm [Fig. 3(a)]. The number of staphylococci ingested by each phagocyte was 27·10 ± 6·2, while intracellular killing, expressed as percentage of killed bacteria to phagocytized bacteria per neutrophil ratio, was 8·4 ± 0·7 [Fig. 3(b)]. Fenoterol at all the concentrations tested did not modify either the proportions of neutrophils ingesting bacteria or the number of bacteria phagocytized by neutrophils or intracellular killing (P>0·05, each comparison) [Fig. 3(a) and 3(b)].

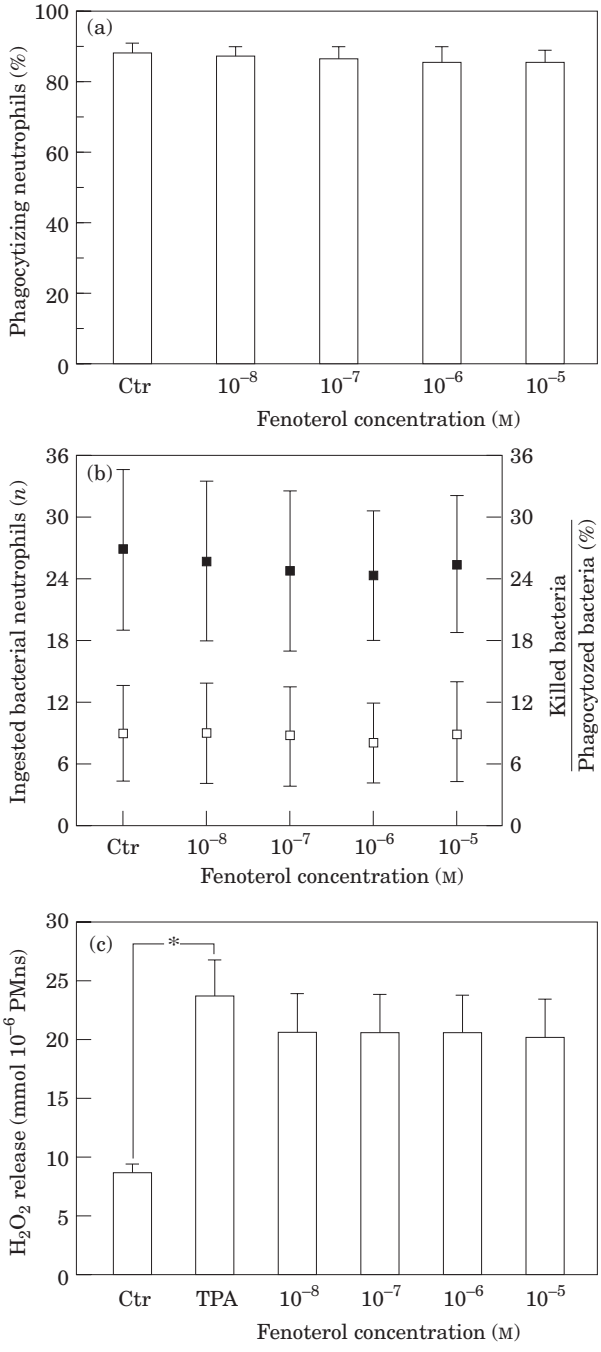


FIG. 3. Antibacterial activity of blood neutrophils. (a) Phagocytozing neutrophils: the percentage of neutrophils ingesting bacteria is expressed on the ordinate, whereas the different experimental conditions, [neutrophils in medium alone (Ctr) and in medium containing different molar concentrations of fenoterol] are expressed on the abscissa. (b) Phagocytosis and intracellular killing: the number of ingested bacteria per cell (■) and the percentage of killed bacteria to phagocytozed bacteria per neutrophil ratio (□) are shown on the ordinates and the different experimental conditions on the abscissa. (c) Hydrogen peroxide (H_2O_2) release: H_2O_2 release is indicated on the ordinate and expressed as nmol produced by 10^6 cells after 60-min incubation at 37°C, while the different experimental conditions are shown on the abscissa [neutrophils incubated in medium alone (Ctr), stimulated with tetradecanoyl-phorbol-acetate (TPA, 100 ng ml^{-1}) in the presence of different molar concentrations of fenoterol]. The data, expressed as mean ± s.e.m., represent the results of 12 experiments. *=P<0·01 *vs.* control.

HYDROGEN PEROXIDE PRODUCTION (H_2O_2)

The cell membrane activator TPA significantly increased H_2O_2 release by neutrophils (P<0·01), [Fig. 3(c)]. This enhanced neutrophil respiratory burst was not modified by the presence of fenoterol at any concentration tested (P>0·05 for each comparison).

Discussion

Evaluation of *in vitro* blood neutrophils from healthy volunteers showed that 1. fenoterol decreases neutrophil locomotion in response to chemotactic signals without modifying cell ability to express adhesion molecules, and 2. it does not influence the antimicrobial activity of neutrophils, i.e. phagocytosis and intracellular killing of bacteria and hydrogen peroxide production.

β_2-adrenoceptor agonists act through interactions with specific receptors located in the plasma membranes of virtually all cell types (27). The binding of agonists to the receptors activates signal transduction mechanisms which produce cellular responses that may be different in the different cell types. In fact, β_2-adrenoceptor agonists raise intracellular cyclic adenosine monophosphate (cAMP) levels by stimulation of adenyl cyclase, and increased cAMP levels have been associated with inhibition of neutrophil function (28–30). In addition, β_2-adrenoceptor agonists may increase or decrease the transcription of genes related to a variety of activities, or may directly modulate receptor expression (27,30). In this context, there is an increasing awareness that activation of a surface receptor may modulate the activity of other receptors, operating at several levels in the signal transduction pathway (27,31).

Previous *in vitro* studies assessing the effects of β-agonists on neutrophil migration and oxygen radical production have reported conflicting results, possibly because of differences in the type of stimulus used to activate neutrophils, in the source of cells (animals *vs*. humans) and in the drugs tested (28,29,32,33). As an example, Ottonello *et al*. showed that salmeterol downregulated *in vitro* the respiratory burst of human neutrophils, while salbutamol had no such effect (17). Although the different activities of these two β_2-agonists may be related to their different pharmacological characteristics (a short- *vs*. a long-acting drug) (33,34), the authors suggested a second hypothesis, that salmeterol-mediated inhibition did not occur at the β_2-receptor level. Indeed, they showed that salmeterol activity was not reversed in the presence of the β-blocker propanolol and did not correlate with the ability of the drug to increase cAMP levels (17). In agreement with the data here presented, preliminary *in vivo* studies suggest that salmeterol is indeed able to reduce neutrophil numbers in patients with neutrophilic airway inflammation (35).

The changes in neutrophil function in response to adrenoreceptor agonists may also be related to modifications of the sub-populations of cells evaluated. Indeed, it was demonstrated *ex vivo* that the respiratory burst of polymorphonuclear leukocytes was increased significantly after i.v. infusion of adrenaline but not of β_1- or β_2-agonists (20). In contrast to β_1- or β_2-agonists, adrenalin, which also activates α-receptors, is able to mobilize the marginating neutrophil pool, which is rich in functionally active cells (20).

There is also evidence that multiple regulatory mechanisms for cAMP may have different effects on cell function, such as adhesion molecule expression. Indeed, in agreement with the results presented here, Derian *et al*. found that isoprenaline was able to downregulate human neutrophil adhesion to human umbilical vein epithelial cells *in vitro* without affecting the expression of adhesion molecules LFA-1 and Mac-1 (30). Interestingly, the expression of LFA-1 and Mac-1 on the neutrophil surface was lowered in the presence of a phosphodiesterase IV inhibitor (rolipram) (30).

We observed that relatively high concentrations of fenoterol (10^{-5} M) induced a dose-dependent inhibition of neutrophil chemotaxis while, still maintaining their antibacterial activity, i.e. that they may influence some neutrophil-induced inflammatory reactions which characterize chronic airway inflammatory disorders without causing significant dysfunction of the host defence mechanism against bacteria. The 'active' concentrations in our experimental system are probably similar to those obtained *in vivo* in clinical practice. Although there are no data on the pharmacokinetics of fenoterol after inhalation from a metred dose inhaler (MDI) or from a nebulizer, some approximations can be made using other β_2-adrenergic drugs. In this context, it has been calculated that after inhalation of 500 μg terbutaline sulphate, the amount of the drug in the large conducting airways is 30–300 ng cm^{-2} and in the small conducting airways 1–30 ng cm^{-2} (generations 9–16) (36,37). This gives approximate drug concentrations of 10^{-4}–10^{-3} M in the fluid of the large airways, and of 10^{-5}–10^{-4} M in the smaller airways (38). Although these concentrations may be higher that those obtained in reality, as they do not take into account uptake of drug from the ciliary fluid layer, the observation by Masclans *et al*. that inhalation of 300 μg of salbutamol inhibits the sequestration of labelled neutrophils in the lungs of healthy subjects after inhalation of platelet-activating factor (39) supports the results of the present study. These authors have suggested that the beneficial role of the drug may be related to its inhibitory activity on airway microvascular leakage (40), however, a direct effect on neutrophil chemotaxis cannot be excluded.

The dowregulation of neutrophil chemotaxis observed here in the presence of fenoterol, although statistically significant, is not impressively so, probably because neutrophils were stimulated with two potent chemotactic stimuli, i.e. LPS and C5a, which are able to increase neutrophil migration by more than 25-fold. These results are, however, in agreement with those reported by Derian *et al*. who, in evaluating the ability of isoprenaline to downregulate human neutrophil adhesion to human umbilical vein epithelial cells *in vitro*, found an inhibitory activity similar to that demonstrated by fenoterol in our study (30).

Finally, a characteristic of many membrane-associated receptors is desensitization after repeated exposure to or high-dose administration of agonists: for example β_2-adrenoceptors on inflammatory cells appear to uncouple rapidly from their intracellular signalling pathways and are eventually lost from the cell surface (27). This *in vitro* effect of fenoterol may therefore not be clinically relevant to the regular treatment of chronic bronchitis patients. However, in agreement with our results, recent clinical trials have shown that β_2-adrenoceptor agonists, when added to other existing regimens, improve the effect of therapy in patients with chronic obstructive pulmonary disease without increasing the frequency of disease exacerbation (41).

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References

1. Thompson AB, Daughton D , Robbins GA, Ghaufouri MA, Oehlerking M, Rennard SI. Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters. *Am Rev Respir Dis* 1989; **140:** 1527–1537.
2. Snider GC. Chronic bronchitis and emphysema. In: Murrey JF, Nadel JA (eds) *Textbook of Respiratory Medicine*. Philadelphia: WB Saunders, 1988: 1069–1106.
3. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989; **320:** 365–376.

4. Hogg JC. Neutrophil traffic. In: Crystal RG, West JB (eds) *The Lung, Vol. 1*. New York, NY: Raven Press 1991: 565–579.
5. Junod AF. Neutrophils, oxidants and tissue damage. In: Pozzi E (ed.) *Neutrophils, Lymphocytes and Lung*. Milan: Masson, 1990: 49.
6. Travis J. Neutrophil enzimes and emphysema. In: Pozzi E (ed.) *Neutrophils, Lymphocytes and Lung.* Milan: Masson, 1990: 49.
7. McElvaney NG, Crystal RG. Proteases and lung injury. In: Crystal RG, West JB (eds) *The Lung, Vol. 2*. New York, NY: Raven Press: 1991: 2205–2218.
8. Stockley RA. Proteolytic enzymes, their inhibitors and lung diseases. *Clin Sci* 1983; **64:** 119–126.
9. Wright DG. The neutrophil as a secretory organ of host defence. In: Gallin J, Fauci AS (eds) *Phagocytic Cells: Advances in Host Defence Mechanisms, Vol. 1*. New York, NY: Raven Press 1982: 75–110.
10. Balbi B, Aufiero A, Pesci A, Oddera S, Zanon P, Rossi GA, Olivieri D. Evaluation of lower respiratory tract inflammation in chronic bronchitis by bronchoalveolar changes associated with local treatment with Immunytal, a biological response modifier. *Chest* 1994; **106:** 19–26.
11. Eller J, Lapa E, Silva JR, Poulter LW, Lode H, Cole PJ. Cells and cytokines in chronic bronchial infection. In: Chignard M, Pretolani M, Renesto P, Vargaftig BB, (eds) Cells and cytokines in lung inflammation. *Ann NY Acad Sci* 1994; **725:** 331–345.
12. Fietta A, Bersani C, De Rose V *et al*. Evaluation of systemic host defence mechanisms in chronic bronchitis. *Respiration* 1988; **53:** 37–43.
13. Riise GC, Ahlstedt S, Larsson S, Enander I, Jones I, Larsson P, Andersson B. Bronchial inflammation in chronic bronchitis assessed by measurement of cell products in bronchial lavage fluid. *Thorax* 1995; **50:** 360–365.
14. Lomas DA, Chambra A, Ip M, Stockley RA. The effects of dexamethasone on human neutrophil functions in vitro and in vivo. *Agents Actions* 1991; **33:** 279–285.
15. Llewellyn-Jones CG, Hill SL, Stockley RA. Effects of fluticasone propionate on neutrophil chemotaxis, superoxide generation and extracellular proteolytic activity in vitro. *Thorax* 1994; **49:** 207–212.
16. Hand WL, Hand DL, King-Thompson NL. Antibiotic inhibition of the respiratory burst response in human polymorphonuclear leukocytes. *Antimicrob Agents Chemother* 1990; **34:** 863–870.
17. Ottonello L, Morone P, Dapino P, Dallegri F. Inhibitory effect of salmeterol on the respiratory burst of adherent neutrophils. *Clin Exp Immunol* 1996; **106:** 97–102.
18. Lofdahl CG, Chung KF. Long acting β_2-adrenoreceptor agonists: a new perspective in the treatment of asthma. *Eur Respir J* 1991; **4:** 218–226.
19. Mita H, Shida T. Anti-allergic activity of formoterol, a new beta-adrenoceptor stimulant, and salbutamol in human leukocytes and human lung tissue. *Allergy* 1983; **38:** 547–553.
20. Burns AM, Keogan M, Domaldson M, Brown DL, Park GR. Effects of inotropes on human leukocyte numbers, neutrophil function and lymphocyte subtypes. *Br J Anaesthesiol* 1997; **78:** 530–535.
21. Gartner I. Separation of human eosinophils in density gradients of polyvinylpyrrolidone-coated silica gel (Percoll). *Immunology* 1980; **40:** 133–136.
22. Oddera S, Silvestri M, Lantero S, Sacco O, Rossi GA. Downregulation of the expression of adhesion molecule (ICAM)-1 on bronchial epithelial cells by fenoterol, a β_2-adrenoceptor agonist. *J Asthma* 1998; **35:** 401–408.
23. Lantero S, Alessandri G, Spallarossa D, Scraso L, Rossi GA. LFA-1 expression by blood eosinophils is increased in atopic asthmatic children and is involved in eosinophil locomotion. *Eur Respir J* 1998; **12:** 1094–1098.
24. Muirhead KA, Schmitt TC, Muirhead AR. Determination of linear fluorescence intensities from flow cytometric data accumulated with logarithmic amplifiers. *Cytometry* 1983; **3:** 251–256.
25. Oddera S, Silvestri M, Sacco O, Eftimiadi C, Rossi GA. N-acetylcysteine enhances in vitro the intracellular killing of *Staphylococcus aureus* by human alveolar macrophages and blood polymorphonuclear leukocytes and partially protects phagocytes from self-killing. *J Lab Clin Med* 1994; **124:** 293–301.
26. Tonetti M, Cavallero A, Botta GA, Niederman R, Eftimiadi C. Intracellular pH regulates the production of different oxigen metabolites in neutrophils: effects of organic acids produced by anaerobic bacteria. *J Leukocyte Biol* 1991; **49:** 180–188.
27. Barnes BJ. Beta-adrenergic receptors and their regulation. *Am J Respir Crit Care Med* 1995; **152:** 828–860.
28. Llewellyn-Jones CG, Stockley RA. The effects of β_2-agonists and methylxantines on neutrophil function in vitro. *Eur Respir J* 1994; **7:** 1460–1466.
29. Rivkin I, Rosenblatt J, Becker EL. The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. The inhibition of neutrophil movement and the evaluation of cyclic AMP levels by catecholamines, prostaglandins, theophylline and cholera toxin. *J Immunol* 1975; **115:** 1126–1134.
30. Derian CK, Santulli RJ, Rao PE, Solomon HF, Barrett JA. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J Immunol* 1995; **154:** 308–317.
31. Oddera S, Silvestri M, Scarso L, Testi R, Rossi GA. Salmeterol inhibits the allergen-induced mononuclear cell proliferation and downregulates GM-CSF release and HLA-DR expression by monocytes. *Pulm Pharmacol* 1997; **10:** 43–49.
32. Nielson CP. β-Adrenergic modulation of the polymorphonuclear leukocyte respiratory burst is dependent upon the mechanism of cell activation. *J Immunol* 1987; **139:** 2392–2397.
33. O'Donnel SR, Wanstall JC. Evidence that the efficacy (intrinsic activity) of fenoterol is higher than that of salbutamol of β-adrenoceptors in guinea-pig trachea. *Eur J Pharmacol* 1978; **47:** 333–341.

34. Lipworth B, Grove A. Evaluation of partial β-receptor agonist activity. *Br J Clin Pharmacol* 1997; **43:** 9–14.
35. Faurschou P, Dahl R, Jeffery P, Venge P, Egerod I. Comparison of the anti-inflammatory effects of fluticasone and salmeterol in asthma: a placebo controlled, double blind, cross-over study with bronchoscopy, bronchial methacholine provocation and lavage. *Eur Respir J* 1997; **10:** 243S (Abstract P1555).
36. Newman SP, Miller AB, Lennard-Jones TR, Moren F, Clarke SW. Improvement of pressurized aerosol deposition with Nebuhaler spacer device. *Thorax* 1984; **39:** 935–941.
37. Newman SP. Therapeutic aerosols. In: Clarke SW, Pavia D (eds) *Aerosols and the Lung in Clinical and Experimental Aspects*. London: Butterworths, 1984; 197–224.
38. Kerrebijn KF. Beta agonists. In: Kaliner MA, Barnes PJ, Persson CGA, (eds) *Asthma: Its Pathology and Treatment*. New York, NY, Maercel Dekker Inc., 1991; 523–545.
39. Masclans JR, Barberà JA, MacNee W, Pavia J, Piera C, Lomena F, Chung KF, Roca J, Rodriguez-Roisin R. Salbutamol reduces pulmonary neutrophil sequestration of platelet-activating factor in humans. *Am J Respir Crit Care Med* 1996; **154:** 529–532.
40. Boschetto P, Roberts NM, Rogers DF, Barnes PJ. Effects of antiasthma drugs on microvascular leakage in guinea pig airways. *Am Rev Respir Dis* 1989; **149:** 416–421.
41. Boyd G, Morice AH, Pounsford JC, Siebert M, Peslis N, Crawford C. An evaluation of salmeterol in the treatment of chronic obstructive pulmonary disease (COPD). *Eur Respir J* 1997; **10:** 815–821.