INTRODUCTION

The mammalian tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are widely distributed throughout the central and peripheral nervous system, where they act as neurotransmitters or neuromodulators. Tachykinins, which are derived from prepro-tachykinins (PTT; there are three different genes, all coding for SP) and are subjected to enzymatic degradation (mainly by angiotensin converting enzyme and by neutral endopeptidase), have been implicated in a large array of biological actions, e.g. pain transmission, neurogenic inflammation, smooth muscle contraction, vasodilation and activation of the immune system.

Their effects are largely mediated via specific G-protein-coupled receptors and at least three subtypes of tachykinin receptors (NK₁, NK₂ and NK₃) have been characterized by the rank order of potency of agonists, by using selective antagonists and by molecular cloning.¹⁻³

The undecapeptide SP, which is synthesized in primary afferent neurons and released from the terminals in response to different stimuli, is known to largely contribute to the local control of the inflammatory and immune responses. It stimulates lymphocyte proliferation and interacts with specific receptors on human T lymphocytes and cultured IM-9 lymphoblasts; degranulates mast cells and induces TNF-α mRNA and TNF-α release from human skin mast cells; modulates human neutrophil activity (by stimulating phagocytosis and...

Summary

Three types of tachykinin receptors, NK₁, NK₂ and NK₃, have been described to preferentially interact with substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) respectively. Experimental evidence indicates that SP and NKA modulate the activity of inflammatory and immune cells, including mononuclear ones, and points to their involvement in lung pathophysiology. We previously reported that NK₁ and NK₂ receptors are present on monocytes (MO) isolated from healthy donors or rheumatoid patients – a greater sensitivity to NK₁ receptor stimulation was observed in the latter condition. This study evaluated the effects of SP and NKA, as well as NK₁ and NK₂ selective agonists and antagonists, on MO obtained from healthy volunteers, healthy smokers or patients with interstitial lung diseases (e.g. sarcoidosis and idiopathic pulmonary fibrosis). Superoxide anion (O₂⁻) production was chosen as a parameter of cell activation. SP and NKA dose-dependently evoked O₂⁻ production from MO in all the conditions evaluated, their effects being competitively antagonized by selective antagonists (CP 96 345 and MEN 10 627, respectively). When selective NK₁ and NK₂ agonists were used, [Sar⁹Met(O₂)¹¹]SP, a selective NK₁ agonist, induced a more than doubled O₂⁻ production in MO obtained from patients with interstitial lung diseases as compared to healthy volunteers, whereas MO isolated from healthy volunteers were more sensitive to NK₂ receptor stimulation. © 2000 Harcourt Publishers Ltd
priming PMN for an enhanced respiratory burst); induces eosinophil activation and fibroblast proliferation; acts synergistically with insulin-like growth factor-1 to enhance corneal epithelial migration; and stimulates guinea-pig peritoneal macrophages as well as rodent and human alveolar macrophages.\(^4\)\(^-\)\(^13\)

Mononuclear phagocytes, either circulating blood monocytes (MO) or tissue macrophages, participate in host defense responses through their capacity to present antigens, to undergo a respiratory burst, to produce and release cytokines, eicosanoids and other soluble mediators. In 1988, it was demonstrated that human blood MO release inflammatory cytokines (such as IL-1, TNF-\(\alpha\) and IL-6) upon challenge in vitro with low concentrations (maximal effects are obtained at 10 nM) of SP, NKA and SP (4–11).\(^14\) SP has been demonstrated to bind human MO/macrophages with high affinity and specificity, \(K_d\) value being in the nanomolar range,\(^15\) even if another group reported that a non-neurokinin SP receptor is present on MO and demonstrated that SP acted only at high micromolar concentrations.\(^16\) More recently, it has been demonstrated that NK\(_2\) receptor expression is increased 4-fold in asthmatics, 3-fold in smokers (HS) (all male, aged between 40 and 45 years).\(^{12}\)

Tachykinins regulate airway smooth muscle cell proliferation,\(^25\) stimulate lung fibroblast proliferation and chemotaxis\(^26\) and induce a respiratory burst in alveolar macrophages from sarcoid patients and healthy smokers.\(^12\)

Pulmonary sarcoidosis is a multisystemic granulomatous disease of unknown etiology mainly affecting lung interstitium, characterized by the presence of multiple non-caseating granulomas. The prominent immunological feature is an accumulation of CD4+ T lymphocytes; alveolar macrophages, derived from circulating MO and accumulated within alveolar structures during sarcoid alveolitis, play a central role in the recruitment and activation of CD4+ lymphocytes.\(^27\) Idiopathic pulmonary fibrosis is an inflammatory-fibrotic disease of unknown etiology, characterized by the accumulation of inflammatory cells in the lower respiratory tract, by alveolar epithelial injury and by progressive interstitial fibrosis\(^28\) in which MO, alveolar macrophages and fibroblasts play a central role, and by releasing oxy-radicals and different cytokines.\(^29\) Macrophage alveolitis in smokers is another condition in which an accumulation of activated mononuclear cells is observed: an increased number of alveolar macrophages, as well as morphophenotypical and functional pattern of macrophages similar to that observed in sarcoidosis, has been documented.\(^30\)\(^,\)\(^31\)

Therefore, we decided to evaluate the effects of SP, NKA and selective receptor agonists and antagonists on MO obtained from healthy volunteers or patients with different lung diseases (e.g. sarcoidosis, idiopathic pulmonary fibrosis, tobacco smoke-related).

**MATERIALS AND METHODS**

**Study population**

Nine patients (four male and five female), aged between 30 and 77 years, with interstitial lung diseases (ILD) were studied: four patients (1 male and 3 female) had an active sarcoidosis (SAR) and five patients (3 male and 2 female) were affected by idiopathic pulmonary fibrosis (IPF). Diagnosis was carried out on the basis of clinical, laboratory and chest X-ray data, and confirmed by histological findings on specimens from transbronchial lung biopsies during bronchoscopy. None of the patients had a medical history of asthma, and pulmonary function tests excluded actual obstruction. No patients had received steroids or other therapy at the time of the study. The other group under study was composed by four healthy smokers (HS) (all male, aged between 40 and 45 years).
Adherent monocytes (0.6–1·10⁶/dish) were washed twice with PBS. non adherent cells (mainly lymphocytes) were removed by 37 °C. Cell suspensions (100 μl) were plated in 6-well tissue culture plates (35 mm diameter, Costar, UK) and allowed to adhere for 90 min at 37°C in a humidified atmosphere containing 5% CO₂. The non adherent cells (mainly lymphocytes) were removed by three gentle washings with PBS.

Isolation of peripheral blood monocytes

Peripheral blood MO were isolated from heparinized venous blood (30–40 ml) by standard techniques of dextran sedimentation (dextran T-500; Pharmacia, UK) and Ficoll-Paque (density = 1.077 g/cm³; Pharmacia) gradient centrifugation (400×g, 30 min) and recovered by thin suction at the interface. Cells were washed twice with balanced phosphate-buffered saline (PBS,) (pH 7.4; Sigma, UK) and resuspended at 1–2×10⁶/ml in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal calf serum (Sigma), 2 mM glutamine (Sigma), 50 μg/ml streptomycin and 5 U/ml penicillin (Sigma). Cell viability, as assessed by trypan blue dye exclusion, was >98%. Purified MO populations were obtained by adhesion (see below) and trypan blue dye exclusion, was >98%. Purified MO populations were obtained by adhesion (see below) and assessed with the pan-macrophage monoclonal antibody anti-CD14 (Becton Dickinson, UK). Cell suspensions (100 μl) were plated in 6-well tissue culture plates (35 mm diameter, Costar, UK) and allowed to adhere for 90 min at 37°C in a humidified atmosphere containing 5% CO₂. The non adherent cells (mainly lymphocytes) were removed by three gentle washings with PBS.

Superoxide anion production

Adherent monocytes (0.6–1×10⁶/dish) were washed twice with PBS and then challenged with increasing concentrations of SP, NKA or selective tachykinin receptor agonists ([Sar²Met(O₂)¹¹]SP: NKα agonist; [β-Ala⁶]-NKA(4–10): NKα agonist) for 30 min. These effects were compared with those evoked by standard stimuli, e.g. the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol 12-myristate 13-acetate (PMA), a direct activator of protein kinase C. In the experiments with tachykinin receptor antagonists (CP 96 345: NKα antagonist; MEN 10 627: NKα antagonist), MO were preincubated for 15 min with these drugs and then challenged with tachykinins.

Superoxide anion production was evaluated by superoxide dismutase (SOD)-inhibitable cytochrome C reduction. The absorbance changes were recorded at 550 nm in a Beckman spectrophotometer and expressed as nmol cytochrome C reduced/10⁶ monocytes/30 min.²¹

Experiments were performed in duplicate and control values (e.g. basal O₂⁻ production) were subtracted from all determinations. All results are expressed as mean ± SEM. Statistical analysis was performed by Student’s t-test.

The apparent affinity (pKₐ) of a given antagonist was calculated as follows: pKₐ = log [DR–1] – log [B], where DR (dose-ratio) was calculated as the concentration of the agonist required in the presence of a given concentration of antagonist to produce the same response as the concentration of the agonist in the absence of the antagonist and [B] was the molar concentration of the antagonist.

Chemicals

The compounds used and their sources were as follows: SP, NKA, [β-Ala⁶]-NKA(4–10), [Sar²Met(O₂)¹¹]SP and CP 96 345 (Peninsula, UK); SOD, PMA, FMLP, PBS, RPMI 1640 and cytochrome C type VI (Sigma, UK). MEN 10 627 was donated from Dr C.A. Maggi, Menarini Laboratories, Firenze, Italy.

RESULTS

Superoxide anion production evoked by standard stimuli

Blood MO were collected from four patients with active sarcoidosis (SAR), five patients with IPF, four HS and five VOL. Basal values (O₂⁻ production from unstimulated MO) were significantly higher in SAR and HS (6.91 ± 1 and 5.97 ± 0.13 nmol cytochrome C reduced/10⁶ MO/30 min respectively, n = 4; P < 0.05) than in VOL (1.32 ± 0.4 nmol cytochrome C reduced/10⁶ MO/30 min; n = 5) or PF (3.19 ± 1.3 nmol cytochrome C reduced/10⁶ MO/30 min; n = 5). These values were subtracted from those observed after challenge with tachykinins or standard stimuli to obtain the net O₂⁻ production. No significant variations among experimental groups were observed after challenge with PMA 10⁻⁷ M (a near maximal concentration): it produced 22.83 ± 0.6, 23.9 ± 1.9, 21.86+2 and 22.5 ± 3 nmol cytochrome C reduced/10⁶ MO/30 min; n = 4–5) in HS, SAR, IPF and VOL, respectively. Similar results were also obtained with the bacterial peptide: FMLP 10⁻⁷ M produced 13.2 ± 0.6, 11.4 ± 0.9, 13.3 ± 0.4 and 8.4 ± 2 nmol cytochrome C reduced/10⁶ MO/30 min in HS, SAR, IPF and VOL respectively.

Superoxide anion production evoked by tachykinins

In the concentration range 10⁻¹²–10⁻⁶ M, the mammalian neuropeptides SP and NKA dose-dependently evoked O₂⁻ production in MO of all the four groups; as expected, maximal activation was achieved at micromolar concentration and was less than those observed by standard stimuli, reaching 3–4 nmol cytochrome C reduced/10⁶ MO/30 min.

At 10⁻⁶ M, NKA produced 2.97 ± 0.26 nmol cytochrome C reduced/10⁶ MO/30 min (n = 4) in cells obtained from HS; 3.56 ± 0.25 nmol cytochrome C reduced/10⁶ MO/30 min (n = 4) in cells obtained from SAR; 3.7±0.2 nmol cytochrome C reduced/10⁶ MO/30 min (n = 4) in cells obtained from IPF and 0.5±0.2 nmol cytochrome C reduced/10⁶ MO/30 min (n = 4) in cells obtained from VOL. Basal values (O₂⁻ production) were significantly higher in SAR and HS (6.91 ± 1 and 5.97 ± 0.13 nmol cytochrome C reduced/10⁶ MO/30 min respectively, n = 4; P < 0.05) than in VOL (1.32 ± 0.4 nmol cytochrome C reduced/10⁶ MO/30 min; n = 5) or PF (3.19 ± 1.3 nmol cytochrome C reduced/10⁶ MO/30 min; n = 5). These values were subtracted from those observed after challenge with tachykinins or standard stimuli to obtain the net O₂⁻ production. No significant variations among experimental groups were observed after challenge with PMA 10⁻⁷ M (a near maximal concentration): it produced 22.83 ± 0.6, 23.9 ± 1.9, 21.86+2 and 22.5 ± 3 nmol cytochrome C reduced/10⁶ MO/30 min; n = 4–5) in HS, SAR, IPF and VOL, respectively. Similar results were also obtained with the bacterial peptide: FMLP 10⁻⁷ M produced 13.2 ± 0.6, 11.4 ± 0.9, 13.3 ± 0.4 and 8.4 ± 2 nmol cytochrome C reduced/10⁶ MO/30 min in HS, SAR, IPF and VOL respectively.
10^6 MO/30 min (n = 4) in SAR; 3.87 ± 0.19 and 3.36 ± 0.5 nmol cytochrome C reduced/10^6 MO/30 min (n = 5) in IPF and VOL respectively. Similar effects, from a quantitative point of view, were determined by SP, which, at 10^-6 M, produced 2.95 ± 0.19, 3.6 ± 0.13, 3.67 ± 0.24 and 3.02 ± 0.2 nmol cytochrome C reduced/10^6 MO/30 min (n = 4–5) in HS, SAR, IPF and VOL respectively.

When selective tachykinin receptor agonists were used, the NK2 selective agonist [b-Ala8]–NKA(4–10) evoked a dose-dependent (10^-12–10^-6 M) respiratory burst and maximal activation in the four groups (2.58 ± 0.19, 3.51 ± 0.24, 3.52 ± 0.27 and 2.89 ± 0.3 nmol cytochrome C reduced/10^6 MO/30 min in HS, SAR, IPF and VOL respectively; n = 4–5) was similar to that of NKA. On the contrary, as depicted in Figure 1, the NK1 selective agonist [Sar9Met(O2)11]SP showed a reduced activity in MO from VOL as compared to other groups: O_2^- production was significantly reduced at all the concentrations evaluated and reached only 1.15 ± 0.12 nmol cytochrome C reduced/10^6 MO/30 min (n = 5) at 10^-6 M (Fig. 1).

The amount of maximal respiratory burst was not significantly varied among groups and agonists (with the only exception of the NK1 selective agonist), while the potency of each agonist was susceptible to important variations. As reported in Table 1, dealing with ED_50 values for mammalian tachykinins and the two selective agonists in all the four groups, significant differences were observed. Monocytes isolated from HS demonstrated a reduced sensitivity to NK2 receptor stimulation as compared to the three other groups: ED_50 values were 2.54 nM for NKA and 8.43 nM for [b-Ala8]–NKA(4–10) in HS to be compared with 0.014 nM and 0.04 nM respectively in VOL (Table 1). Although less pronounced, differences were also recorded among groups for NK1 receptor stimulation (Table 1).

### DISCUSSION

This study demonstrates that mammalian tachykinins SP and NKA, as well as the selective NK1 agonist [Sar9Met(O2)11]SP and the NK2 selective agonist [b-Ala8]–NKA(4–10), induce O_2^- production in MO obtained from human volunteers, healthy smokers or patients with ILD. All agonists act dose-dependently: maximal effects are observed at micromolar concentrations, but the respiratory burst is just detectable at concentrations as low as 10^-12–10^-11 M. Selective NK1 and NK2 agonists competitively antagonize tachykinin-evoked O_2^- production from MO, further confirming the involvement of NK, and NK2 receptors.

However, according to the clinical situation of the donor (healthy volunteer, healthy smoker, patient with active sarcoidosis or patient with idiopathic lung fibrosis), some variations are observed. Since circulating MO are the precursors of alveolar macrophages, the most abundant inflammatory cell type in the lung, an altered

![Fig. 1](http://example.com/fig1.png)

**Fig. 1** Effects of the NK1 selective agonist [Sar9Met(O2)11]SP on superoxide anion production in monocytes from different patients: healthy smokers ( ), patients with active sarcoidosis (■), patients with idiopathic pulmonary fibrosis ( ). Mean ± SEM; n = 4–5. * P < 0.05.

<table>
<thead>
<tr>
<th>Agonists</th>
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<tr>
<td>NKA</td>
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</tr>
<tr>
<td>[b-Ala8]–NKA(4–10)</td>
<td>8.43</td>
</tr>
<tr>
<td>SP</td>
<td>1.43</td>
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<tr>
<td>[Sar9Met(O2)11]SP</td>
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<tr>
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<td>5.53</td>
<td>1.8</td>
<td>2.15</td>
</tr>
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HS: healthy smokers; SAR: sarcoidosis; IPF: idiopathic pulmonary fibrosis; VOL: healthy volunteers.
responsiveness of these cells might exert a profound influence at pulmonary level.

As a general rule, and with only the exception of [Sar\textsuperscript{2},Met(O\textsubscript{2})\textsuperscript{11}]SP, the efficacy (evaluated by measuring maximal O\textsubscript{2}\textsuperscript{−} production) of the agonists used is similar, while the potency (that is, the ED\textsubscript{50} of the different drugs) varies considerably, according to the clinical condition. Alveolar macrophages obtained from patients with sarcoidosis or IPF are known to have a higher respiratory burst than MO from the same individuals; but it has also been reported that MO from patients usually produce per se (that is, in the absence of added stimuli) more elevated oxy-radicals.\textsuperscript{32}

Tobacco smoke greatly affects MO responsiveness: smokers have a greater O\textsubscript{2}\textsuperscript{−} release\textsuperscript{33} and a depressed capacity to release cytokines, including TNF-\alpha, than non-smokers.\textsuperscript{34} Cigarette smoke induces the surface expression of cell adhesion molecules such as ICAM-1, ELAM-1 and VCAM-1 and favours transendothelial migration of MO.\textsuperscript{35} In rodents, tobacco smoke enhances airway responsiveness to SP, mainly by inactivating neutral endopeptidase,\textsuperscript{36} stimulating primary afferent sensory nerves and releasing tachykinins in the lung.\textsuperscript{37,38}

However, according to our results, MO from healthy smokers present a reduced sensitivity to NK, and NK\textsubscript{2} receptor stimulation (as compared to MO from volunteers or ILD patients), as evidenced by the higher ED\textsubscript{50} values: we have no definite explanation for this fact, but some attempts can be afforded. Endogenous SP (about 20–50 pg/3\times10\textsuperscript{6} cells) is released by freshly isolated human blood MO and 7 to 10 day cultured macrophages;\textsuperscript{17} cigarette smoke could potentiate this SP release (as it occurs in guinea-pig lung),\textsuperscript{39} probably leading to desensitization and/or down-regulation.

Mutual interactions between TNF-\alpha and tachykinins have been described in different models\textsuperscript{39} and TNF-\alpha was shown to induce SP expression by sympathetic ganglia;\textsuperscript{40} since TNF-\alpha is reduced in MO from smokers,\textsuperscript{41} this cytokine could be implicated. Moreover, a recent paper by Emmms and Rogers\textsuperscript{41} demonstrates that, in the guinea-pig, cigarette smoke exposure reduced the magnitude of subsequent bronchoconstriction induced by NKA by 40%, in keeping with the results reported here.

Conversely, if MO are capable to release SP (as well as to be activated by neurokinins) and this tachykinin, together with NKA, induces airway smooth muscle and fibroblast proliferation, important effects on ILD could be envisaged. As a matter of fact, MO obtained from patients with sarcoidosis or IPF present (these data) a good sensitivity to both NK, and NK\textsubscript{2} receptor stimulation.

It is also worth noting that the apparent affinities (pK\textsubscript{a}) of the two selective antagonists measured in our experiments (around 10) are higher than the corresponding affinities measured in other models;\textsuperscript{1} however, this is not surprising, because the affinities of natural agonists, too, are elevated. Furthermore, such a situation has been previously documented in guinea-pig alveolar macrophages, which are largely derived from circulating MO.\textsuperscript{22} In conclusion, these data demonstrate that NK, and NK\textsubscript{2} receptor stimulation trigger superoxide anion production in MO from healthy donors, healthy smokers and patients with ILD.

The small number of patients evaluated for each clinical condition does not allow a definitive appraisal: more experiments are required to do so. However, these data suggest that sensitivity to a given tachykinin could vary according to different clinical conditions.

ACKNOWLEDGEMENTS

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