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Characterization of the receptor and the mechanisms underlying the inflammatory response induced by des-Arg^9-BK in mouse pleurisy

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1 The characterization of the B_1 kinin receptor, and some mediators involved in the inflammatory response elicited by intrathoracic (i.t.) administration of des-Arg^9-bradykinin (BK) in the mouse model of pleurisy, was investigated.

2 An i.t. injection of des-Arg^9-BK (10–100 nmol per site), a selective B_1 agonist, caused a significant and dose-related increase in the vascular permeability observed after 5 min, which peaked at 1 h, associated with an increase in cell influx, mainly neutrophils, and, to a lesser extent, mononuclear cell influx, peaking at 4 h and lasting for up to 48 h. The increase in fluid leakage caused by des-Arg^9-BK was completely resolved 4 h after peptide injection. I.t. injection of Lys-des-Arg^9-BK (30 nmol per site) caused a similar inflammatory response.

3 Both the exudation and the neutrophil influx elicited by i.t. injection of des-Arg^9-BK were significantly antagonized ($P<0.01$) by an i.t. injection of the selective B_1 antagonists des-Arg^9-[Leu^8]-BK (60 and 100 nmol per site) or des-Arg^9-NPC 17731 (5 nmol per site), administered in association with des-Arg^9-BK ($P<0.01$), or 30 and 60 min before the cellular peak, respectively. In contrast, an i.t. injection of the B_2 bradykinin selective receptor antagonist Hoe 140 (30 nmol per site), at a dose which consistently antagonized bradykinin (10 nmol per site)-induced pleurisy, had no significant effect on des-Arg^9-BK-induced pleurisy.

4 An i.t. injection of the selective tachykinin receptor antagonists (NK_1) FK 888 (1 nmol per site), (NK_2) SR 48968 (20 nmol per site) or (NK_3) SR 142801 (10 nmol per site), administered 5 min before pleurisy induction, significantly antagonized neutrophil migration caused by i.t. injection of des-Arg^9-BK. In addition, FK 888 and SR 142801, but not SR 48968, also prevented the influx of mononuclear cells in response to i.t. injection of des-Arg^9-BK ($P<0.01$). However, the NK_3 receptor antagonist SR 142801 (10 nmol per site) also significantly inhibited des-Arg^9-BK-induced plasma extravasation. An i.t. injection of the calcitonin gene-related peptide (CGRP) receptor antagonist $CGRP_{8-37}$ (1 nmol per site), administered 5 min before pleurisy induction, inhibited des-Arg^9-BK-induced plasma extravasation ($P<0.01$), without significantly affecting the total and differential cell migration.

5 The nitric oxide synthase inhibitors L-NOARG and L-NAME (1 pmol per site), administered 30 min beforehand, almost completely prevented des-Arg^9-BK (i.t.)-induced neutrophil cell migration ($P<0.01$), and, to a lesser extent, mononuclear cell migration ($P<0.01$). The D-enantiomer D-NAME had no effect on des-Arg^9-BK-induced pleurisy. At the same dose range, L-NOARG and L-NAME inhibited the total cell migration ($P<0.01$). L-NAME, but not L-NOARG caused significant inhibition of des-Arg^9-BK-induced fluid leakage. Indomethacin (1 mg kg^{-1}, i.p.), administered 1 h before des-Arg^9-BK (30 nmol per site), inhibited the mononuclear cell migration ($P<0.05$), but, surprisingly, increased the neutrophil migration at 4 h without interfering with plasma extravasation. The administration of terfenadine (50 mg kg^{-1}, i.p.), 30 min before des-Arg^9-BK (30 nmol per site), did not interfere significantly with the total cell migration or with the plasma extravasation in the mouse pleurisy caused by i.t. injection of des-Arg^9-BK.

6 Pretreatment of animals with the lipopolysaccharide of *E. coli* (LPS; 10 μg per animal, i.v.) for 24 h did not result in any significant change of the inflammatory response induced by i.t. injection of des-Arg^9-BK compared with the saline treated group. However, the identical treatment of mice with LPS resulted in a marked enhancement of des-Arg^9-BK induced paw oedema ($P<0.01$).

7 In conclusion, we have demonstrated that the inflammatory response induced by i.t. injection of des-Arg^9-BK, in a murine model of pleurisy, is mediated by stimulation of constitutive B_1 receptors. These responses are largely mediated by release of neuropeptides such as substance P or CGRP and also by NO, but products derived from cyclo-oxygenase pathway and histamine seem not to be involved. Therefore, these results further support the notion that the B_1 kinin receptor has an important role in modulating inflammatory responses, and it is suggested that selective B_1 antagonists may provide therapeutic benefit in the treatment of inflammatory and allergic conditions.

Keywords: B_1 and B_2 agonists and antagonists; neuropeptide antagonists; nitric oxide; pleurisy; inflammation

Introduction

Bradykinin (BK) and lysil-bradykinin (LBK) are proinflammatory and algesic peptides, generated in plasma and peripheral tissues after trauma or infection from low and high molecular weight kininogens, respectively, by the action of serine protease kallikreins. The effects of kinins involve the activation of two membrane receptors, B_1 and B_2. The B_2 receptors exhibit higher affinity for BK, are present in peripheral and central nervous systems, and are normally

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responsible for the maintenance of most physiological kinin effects. On the other hand, the B_1 kinin receptors are only rarely present in non-traumatized tissues, and show greater affinity for the kininase I active metabolites des-Arg^9-BK and Lys-des-Arg^9-BK than for BK itself (Regoli & Barabé, 1980; Marceau, 1995; Bhoola *et al*., 1992; Farmer & Burch, 1992; Hall, 1992; Dray & Perkins, 1993). The B_1 receptors may be up-regulated following tissue trauma or infection, or by a variety of treatments *in vivo*, including treatment with endotoxin, or cytokines, or after desensitization of B_2 receptors, or long-term treatment with *Mycobacterium bovis* Calmette-Guérin (BCG) (Davis & Perkins, 1994a,b; Perkins & Kelly, 1993; Campos *et al*., 1995; 1996; 1997).

Evidence now suggests that B_1 receptors could have a relevant role in certain pathological conditions, mainly in the control of inflammatory processes (Farmer & Burch, 1992; Hall, 1992; Marceau, 1995). Both types of kinin receptor have been cloned in a variety of animal species and they are founder members of the seven transmembrane G protein family of receptors, sharing great sequence homology at the amino acid level (McEachern *et al*., 1991; Hess *et al*., 1992; 1994; Menke *et al*., 1994; Ma *et al*., 1994; Pesquero *et al*., 1996). However, compared with B_2 receptors, much less is known about the BK B_1 receptor subtypes, principally their physiological and pathological relevance in controlling the inflammatory processes (see for review Marceau & Regoli, 1991; Marceau, 1995).

The aim of this study was to investigate, through the use of highly selective kinin antagonists, the possible proinflammatory role exerted by the selective B_1 agonist des-Arg^9-BK in a murine model of pleurisy. Additionally, we tried to characterize some of the mechanisms involved in its inflammatory effect.

Methods

Animals

Male Swiss mice (weighing 20–25 g) from our own colony were used in this study. The animals were maintained in an environment of controlled temperature ($21 \pm 2°C$), illuminated by daylight supplemented by electric light, from 6 h 00 min to 18 h 00 min. Food and water were provided *ad libitum*.

Induction of pleurisy

Pleurisy was induced by intrathoracic (i.t.) injection of 100 μl of sterile 0.9% NaCl w/v solution, containing des-Arg^9-BK (10 to 100 nmol per site), into the right pleural space through the chest skin, according to the previously-described technique of Henriques *et al*.(1990) and Saleh *et al*. (1996). An equal volume of a sterile solution of 0.9% of NaCl w/v was injected i.t. into the control animals.

Exudate quantification

Mice were injected intravenously with a solution of Evans blue dye (25 mg kg^{-1} in 0.2 ml, i.v.) 24 h before being injected i.t. with the agonist. After i.t. injection of des-Arg^9-BK, the animals were killed at different periods of time (5 min to 72 h) with an overdose of ether, and their thoracic cavities were opened and washed with 1 ml of phosphate buffered solution (PBS–pH 7.4; of the following composition (mM): NaCl 137, KCl 2 and phosphate buffer 10) containing heparin (20 iu ml^{-1}). The volume was collected with automatic pipettes and cells were removed by centrifugation. After this, samples of 500 μl were stored in the freezer ($-20°C$) to determine the concentration of Evans blue dye. On the day of experiments, a batch of samples was defrosted to room temperature and the amount of dye was evaluated by obtaining the absorbance values at 600 nm with a Hitachi U-2001 spectrophotometer, by interpolation from the standard curve of Evans blue in the range 0.5–10 μg ml^{-1}.

Leukocyte counts

Total leukocyte counts were performed in Neubauer chambers by means of an optical microscope, after the pleural fluid had been diluted in Türk solution (1 : 20). Differential cell counts were performed after cytocentrifugation (Cytospin 3) and staining with May-Greenwald-Giemsa, and the analysis was carried out under immersion objective. Around 300 cells were counted, the results being expressed as the number of each cell population ml^{-1}.

Experimental procedures

The inflammatory response induced by des-Arg^9-BK (10 to 100 nmol per site) in the pleural space of the mice was characterized by an increase in exudation observed after 5 min, peaking at 1 h. This effect was associated with an increase in total cells which peaked at 4 h and lasted for up to 48 h. Thus, these periods of time were chosen in order to analyse the mediators involved in des-Arg^9-BK or Lys-des-Arg^9-BK-mediated inflammatory response. Protocols for drug administration and intervals before des-Arg^9-BK i.t. injection were as follows: des-Arg^9-[Leu^8]-BK (60 and 100 nmol per site, a B_1 antagonist), des-Arg^9-NPC 17731 (5 nmol per site, a B_1 antagonist) (Campos *et al*., 1996) and Hoe 140 (30 nmol per site, a B_2 antagonist) were administered in association with des-Arg^9-BK for measure of exudation, or at 30 or 60 min before the peak neutrophil response, in order to analyse the polymorphonuclear infiltration induced by des-Arg^9-BK; the selective tachykinin receptor antagonists NK_1 (FK 888, 1 nmol per site), NK_2 (SR 48968, 20 nmol per site), NK_3 (SR 142801, 10 and 30 nmol per site) and the receptor antagonist of the $CGRP_1$-receptors ($CGRP_{8-37}$, 1 nmol per site), were all administered by the i.t. route 5 min before i.t. injection of des-Arg^9-BK (both for exudation and polymorphonuclear peaks). The nitric oxide synthase inhibitors, N^G-nitro-L-arginine (L-NOARG), L-NAME (N^{ω}-nitro-L-arginine methyl ester), and D-NAME (N^{ω}-nitro-D-arginine methyl ester) each 1 pmol per site, were administered i.t. 30 min before des-Arg^9-BK, for their effects on both peak responses.

To assess the possible induction of B_1 receptors, animals were pretreated with 10 μg per animal of LPS (lipopolysaccharide of *Escherichia coli*) administered intravenously (i.v.), 24 h before pleurisy induction, as described previously (Cabrini *et al*., 1996; Campos *et al*., 1996). The role of cyclooxygenase products derived from arachidonic acid pathway and histamine in des-Arg^9-BK-induced pleurisy was also investigated. For this purpose animals were treated with indomethacin (1 mg kg^{-1}, i.p.) and terfenadine (50 mg kg^{-1}, i.p.) 1 h before the i.t. injection of des-Arg^9-BK for effects on both peak responses.

Mouse hindpaw oedema

In order to determine the effectiveness of LPS treatment on upregulation of responses to des-Arg^9-BK, separate groups of mice received an i.v. injection of LPS (10 μg per animal 24 h previously). Control animals received a similar volume of

sterile saline (0.1 ml per animal i.v., 24 h previously). Twenty four hours after the saline or LPS injection, animals were anaesthetized with ether and received a subcutaneous injection of des-Arg^9-BK (50 nmol per paw) in the right paw. The contralateral paw received the same volume of sterile saline and served as a control. Oedema was measured at several point times (10, 20, 30, 60 and 120 min) by use of a plethysmometer, as described previously Campos *et al*., 1996). Oedema was expressed as the difference in millilitres between the test and control paws.

Drugs

The following drugs were used: L-NOARG (N^G-nitro-L-arginine), L-NAME (N$^\omega$-nitro-L-arginine methyl ester), D-NAME (N$^\omega$-nitro-D-arginine methyl ester), indomethacin, CGRP$_{8-37}$, Evans blue, terfenadine and lipopolysaccharide (*Escherichia coli* serotype 026 : B6), all from Sigma Chemical Company (St. Louis, U.S.A.), heparin (Liquemine, Roche, Brazil), May Greenwald-Giemsa (Labormed, Brazil), des-Arg^9-BK, Lys-des-Arg^9-BK and des-Arg^9-[Leu^8]-BK (Peninsula Belmont, CA, U.S.A.), Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) and des-Arg^9-NPC 17731 (D-Arg-Arg-Pro-Hyp - Gly - Phe - Ser-D-Hyp-(transpropyl) -Oic), which were kindly supplied by the Department of Pharma Synthesis of Hoechst (Frankfurt Main, Germany) and by SCIOS/NOVA. (Baltimore, U.S.A.), respectively; FK 888 (94**R**)-4-hidroxi-1-([1 - metil - 1h - indol - 3-il]carbonil-)-L-N-benzil-N-metil-3-2-(2-naftil)-L-alaninamide) was kindly supplied by Fujisawa Pharmaceutical CO (Osaka, Japan); SR 48968 ((**S**)-N-metil-N-[4 - acetylamino -4-phenilpiperidino-2-(3,4-diclorophenil)butil]benzamide) and SR 142801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichloro-phenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methylacetamida) were kindly supplied by Sanofi Recherche (Montpellier, France). The stock solutions for all peptides were prepared in PBS (1 – 10 mM), except FK 888, SR 48968 and SR 142801 which were dissolved in absolute ethanol in siliconized plastic tubes. Indomethacin was dissolved in 5% $NaHCO_3$ solution. All drugs were maintained at -18°C and diluted to the desired concentration just before use. Other drugs were prepared daily in sterile saline solution (NaCl 0.9%). The final concentration of ethanol was less than 10%, and caused no effect *per se* and did not affect des-Arg^9-BK pro-inflammatory effects.

Statistical analysis

Data are presented as mean $\pm$ s.e.mean. Differences between groups were determined by analysis of variance (ANOVA) followed by Dunnett's test or by Student's unpaired t test when necessary, and were considered to be statistically significant when P was less than 0.05 ($P<0.05$).

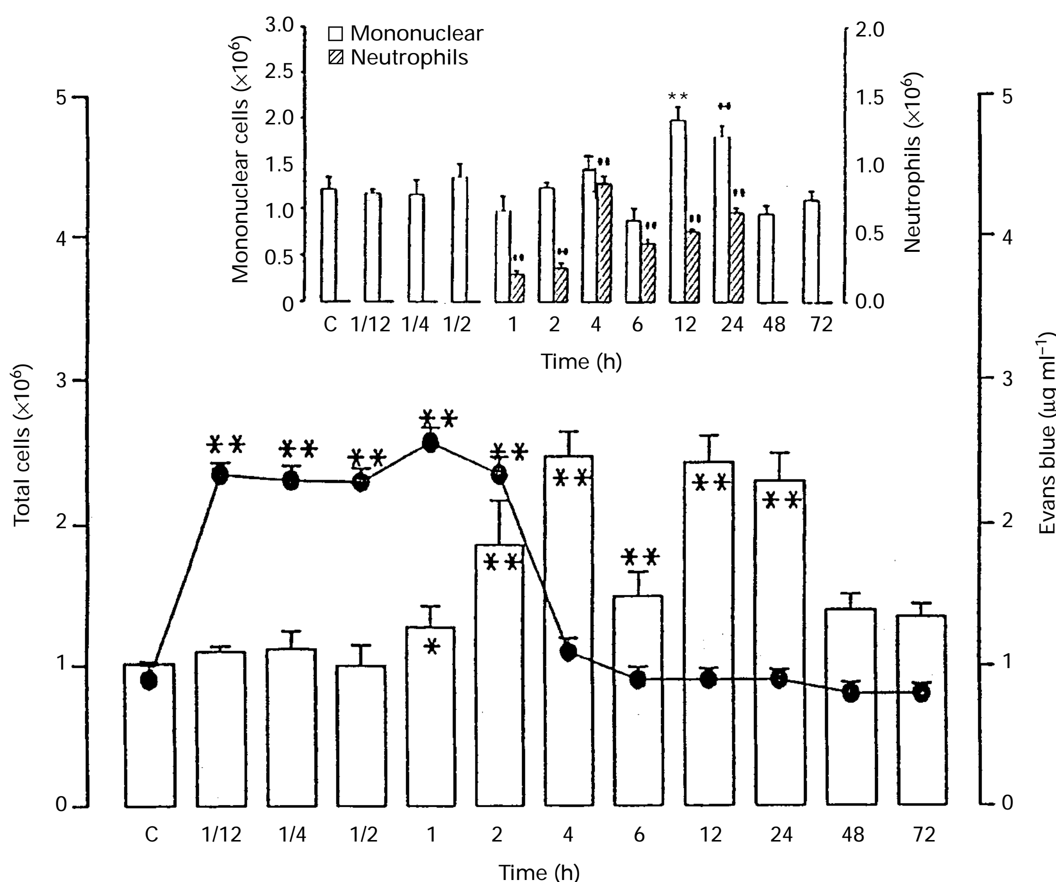

Figure 1 Time course profile of total cell content and Evans blue extravasation induced by des-Arg^9-BK (30 nmol per site) in the pleural cavity of mice. Control response (C) obtained in animals injected only with sterile saline. Asterisks inside and outside the columns indicate the statistical significance of both total cell content (columns) and exudate values (solid circles) in comparison to the respective control values (C). The inset shows the variation of both mononuclear cells and neutrophils with time, in the mouse pleurisy model, induced by des-Arg^9-BK. Asterisks inside and outside the columns indicate statistically significant differences for both neutrophils and mononuclear cells in comparison to control animals. Each column and symbol represent the mean of 5 to 10 animals with s.e.means also shown. In some groups, the s.e.mean values were smaller than the size of the symbol. *$P<0.05$ and **$P<0.01$.

Results

Kinetics of cells induced by des-Arg^9-BK and Lys-des-Arg^9-BK in mouse pleural cavity

Figure 1 shows that i.t. injection of the selective B_1 agonist des-Arg^9-BK (30 nmol per site) caused a time-dependent increase in fluid leakage and cell infiltration which followed a different time course. This plasma extravasation, starting at 5 min, peaked at 1 h ($P<0.05$) and decreased after 2 h ($P<0.05$), while polymorphonuclear neutrophil cell migration was evident after 1 h and peaked at 4 h after des-Arg^9-BK challenge ($P<0.01$). The leukocyte influx suddenly decreased at 6 h after pleurisy induction, but thereafter increased for up to 24 h ($P<0.01$). At 48 and 72 h after des-Arg^9-BK-induced pleurisy, the polymorphonuclear cells had returned to their normal values ($P>0.05$). Figure 1 (inset) shows that neutrophil influx corresponds to about 50% of the total cell migration in response to i.t. injection of des-Arg^9-BK. Neutrophil influx increased after 1 h ($P<0.01$) and peaked at 4 h, remaining significantly high for up to 24 h after pleurisy induction. However, the mononuclear cells were augmented significantly after 12 h of pleurisy induction, remaining high for up to 12 h ($P<0.01$). Both the neutrophil and mononuclear cell migration decreased to the control values 48 h after pleurisy induction ($P>0.05$). Figure 2 (a, b and c) shows that the i.t. injection of the other selective B_1 agonist Lys-des-Arg^9-BK (30 nmol per site) was also able to induce a marked increase in plasma exudation, peaking at 1 h, and mononuclear and neutrophil cells migration, peaking at 4 h, in the mouse pleural cavity. Neither cell migration nor plasma extravasation in response to i.t. injection of Lys-des-Arg^9-BK differed significantly when compared to that of the response induced by des-Arg^9-BK.

The i.t. injection of des-Arg^9-BK (10, 30 and 100 nmol per site) caused a dose-related increase in the total cell migration (control response: $1.1\times10^6\pm0.11$ versus $1.65\times10^6\pm0.12$; $3.06\times10^6\pm0.14$; $4.3\times10^6\pm0.13$ in the presence of 10, 30 and 100 nmol per site of des-Arg^9-BK, respectively, $n=6$). Figure 2d shows that des-Arg^9-BK (30 and 100 nmol per site) also increase significantly the plasma exudation, although this effect was not dose-dependent. In addition, des-Arg^9-BK increased dose-dependently both the mononuclear and neutrophil cells (Figure 2e and f). However, while exudation reached a maximum at 30 nmol per site of des-Arg^9-BK, the maximal response for des-Arg^9-BK-induced mononuclear and neutro-

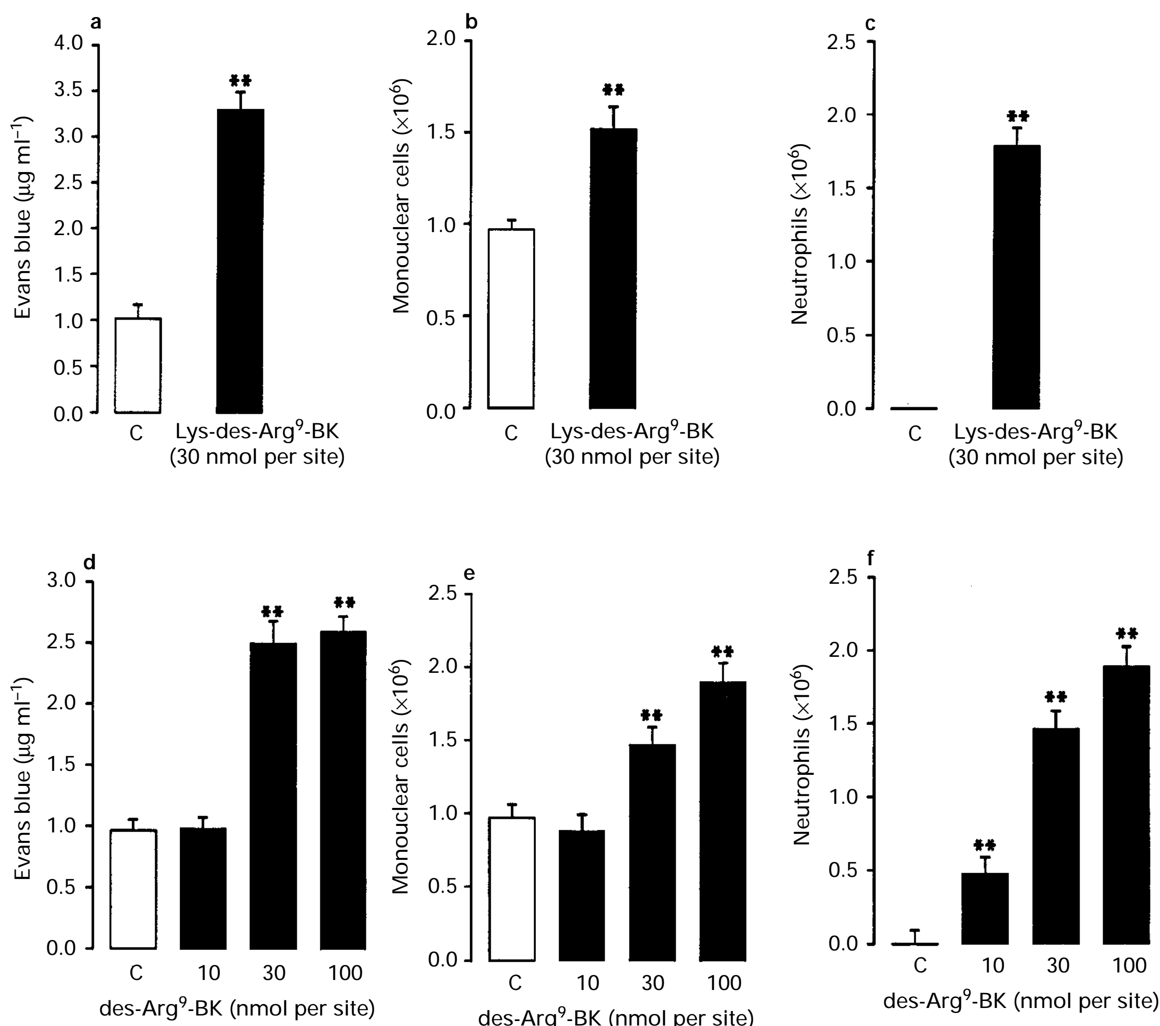

Figure 2 (a) Evans blue extravasation, (b) mononuclear cells and (c) neutrophils induced by Lys-des-Arg^9-BK (30 nmol per site) in the pleural cavity of mice. (d–f) Dose-response profile of (d) Evans blue extravasation, (e) mononuclear cells and (f) neutrophils induced by des-Arg^9-BK (10 to 100 nmol per site) in the pleural cavity of mice. Control responses (C) were obtained in animals injected only with sterile saline. Asterisks above each column indicate statistically significant differences in comparison with control animals. Each column represents the mean of 5 to 6 animals with s.e.means also shown. $*P<0.05$ and $**P<0.01$.

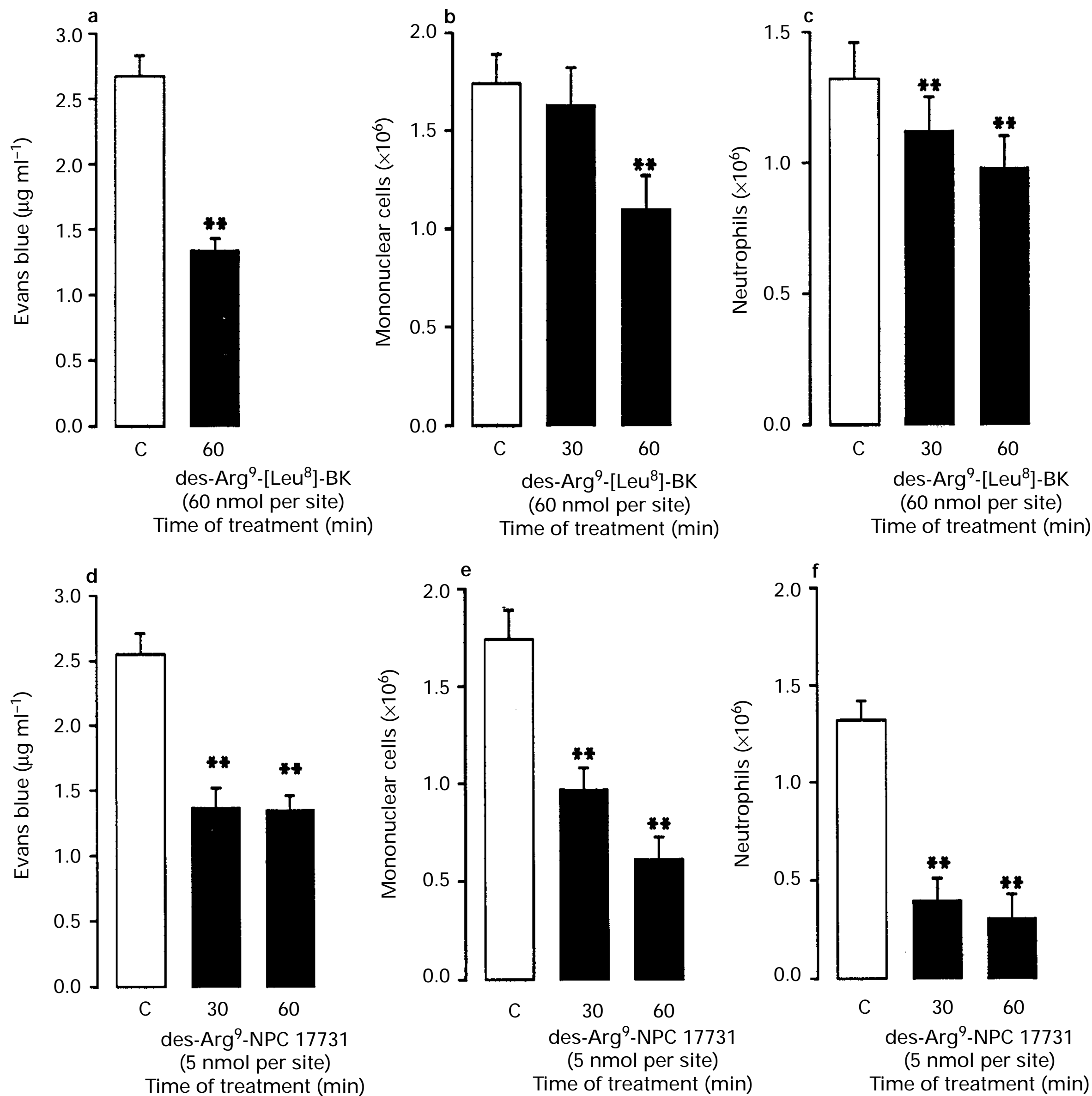

Figure 3 The content of Evans blue extravasation (a, d), the mononuclear cells (b, e) and neutrophils (c,f) induced by i.t. injection of des-Arg⁹-BK (30 nmol per site) in the pleural cavity of the mice in the absence (open columns) and presence (solid columns) of the i.t. injection of either (a,b,c) des-Arg⁹-[Leu⁸]-BK (60 nmol per site) or (d,e,f) des-Arg⁹-NPC 17731 (5 nmol per site) administered 30 and 60 min previously, for cellular peakage (4 h), or by co-injection for plasma leakage (1 h). Asterisks in each column indicate statistically significant differences compared with control animals. Each column represents the mean of 5 to 6 animals, with s.e.means also shown. $*P<0.05$ and $**P<0.01$.

phils infiltration could not be reached, even after i.t. injection of a high concentration of this peptide (100 nmol per site) (Figure 2e and f).

Effect of the B_1 and B_2 selective antagonists on des-Arg⁹BK-induced pleurisy in mice

Figure 3 (a, b and c) illustrates that the plasma exudation, mononuclear and neutrophil cells induced by i.t. injection of des-Arg⁹-BK (30 nmol per site) were inhibited significantly by des-Arg⁹-[Leu⁸]-BK (60 nmol per site), when the animals were treated 30 or 60 min before the peak cell responses. The inhibitions caused by des-Arg⁹-[Leu⁸]-BK (60 and 100 nmol per site) were the following: $52.0\pm1.6\%$ and $49.8\pm1.9\%$, for plasma leakage (Figure 4a); $6.4\pm1.1\%$ at 30 min and $36.8\pm1.2\%$ ($P<0.01$) at 60 min for des-Arg⁹-[Leu⁸]-BK at 60 nmol per site (Figure 3b); $52.1\pm1.3\%$ for des-Arg⁹-[Leu⁸]-BK at 100 nmol per site at 60 min (Figure 4b), ($P<0.01$) for mononuclear cells; $15.0\pm1.3\%$ at 30 min and $25.8\pm1.3\%$ at 60 min for des-Arg⁹-[Leu⁸]-BK 60 nmol per site (Figure 3c) ($P<0.01$), and $38.9\pm1.1\%$ for des-Arg⁹-[Leu⁸]-BK 100 nmol per site at 60 min (Figure 4c) for neutrophils.

Very similar inhibition of des-Arg⁹-BK-induced pleurisy was observed when animals were treated 30 or 60 min before the cell peaks with the new B_1 selective receptor antagonist des-Arg⁹-NPC 17731 (5 nmol per site) (Figure 3d,e,f). Inhibition of the total cell influx (mean±s.e.mean) was: $57.8\pm1.5\%$ and $71.2\pm2.0\%$, (results not shown), for mononuclear cell was $44.3\pm2.3\%$ and $64.6\pm2.2\%$ (Figure 3e), and $69.7\pm1.1\%$ and $76.6\pm2.0\%$ for neutrophil influx at 30 and 60 min of pretreatment, respectively (Figure 3f) ($P<0.01$). Inhibition of the exudation caused by des-Arg⁹-NPC 17731 (5 nmol per site) was $46.3\pm1.5\%$ (Figure 3d). Injection of higher concentration of des-Arg⁹-NPC 17731 (20 nmol per site) resulted in a marked

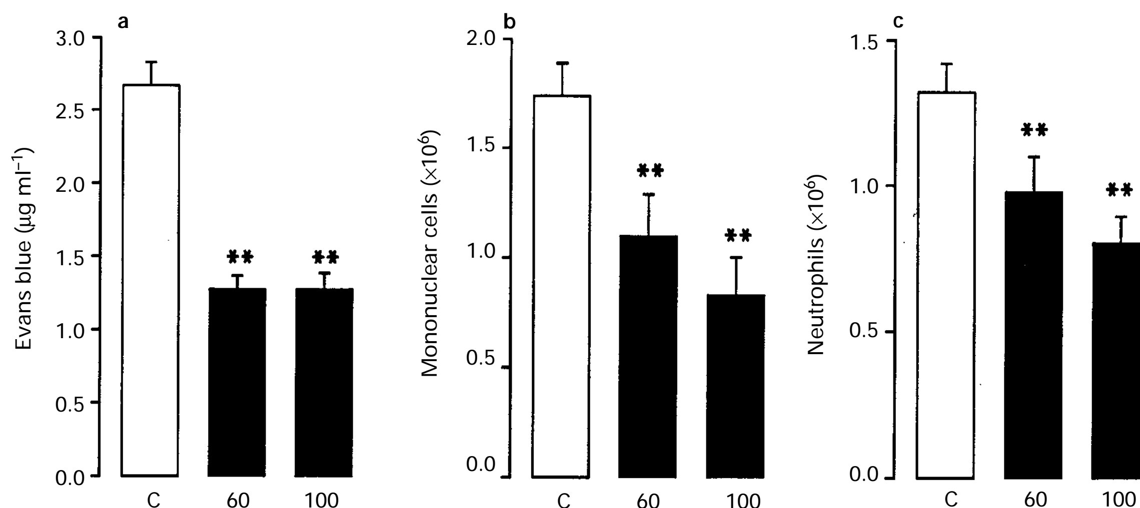

Figure 4 The content of Evans blue extravasation (a), the mononuclear cells (b), and neutrophils (c) induced by des-Arg^9-BK (30 nmol per site) in the pleural cavity of the mice in the absence (open columns) or presence (solid columns) of des-Arg^9-[Leu^8]-BK (60 and 100 nmol per site) administered 60 min before peak cell response (4 h) or by co-injection for plasma leakage (1 h). Asterisks in each column indicate statistically significant differences in comparison to control animals. Each column represents the mean of 5 to 6 animals with s.e.means also shown. $^*P<0.05$ and $^{**}P<0.01$.

potentiation of the effects of des-Arg^9-BK on all the parameters studied (results not shown).

In contrast, an i.t. injection of Hoe 140 (30 nmol per site) given 30 min previously, at a dose which has been demonstrated in a previous experiment to inhibit the inflammatory response induced by bradykinin (10 nmol per site) by 70% (results not shown), did not inhibit significantly either the plasma extravasation or the polymorphonuclear cell influx caused by i.t. injection of des-Arg^9-BK in mice (exudation: control response 2.5 ± 0.12 μg ml^{-1} versus 2.6 ± 0.14 μg ml^{-1} in the presence of Hoe 140; mononuclear cells: control response $1.72\times10^6\pm0.13$ cells versus $1.61\times10^6\pm0.11$ cells in the presence of Hoe 140; neutrophils: control response $1.32\times10^6\pm0.13$ cells versus $1.28\times10^6\pm0.12$ cells in the presence of Hoe 140, $n=6$ per group). The i.t. injection of des-Arg^9-[Leu^8]-BK (60 and 100 nmol per site), des-Arg^9-NPC 17731 (5 nmol per site) or Hoe 140 (10 nmol per site) did not cause any significant effect *per se* on total cell migration or on increase of vascular leakage compared with that of saline-treated animals ($n=3$, results not shown).

Involvement of sensory neuropeptides on des-Arg^9-BK-induced pleurisy

Figure 5 (a, b and c) shows that i.t. injection of the selective antagonists of NK_1 (FK 888, 1 nmol per site), NK_2 (SR 48968, 20 nmol per site) or NK_3 (SR 142801, 10 nmol per site) receptors, all administered 5 min before pleurisy induction, antagonized, by $72.6\pm2.1\%$, $62.9\pm1.8\%$ and $86.7\pm1.8\%$, the neutrophil cell migration ($P<0.01$) in response to i.t. injection of des-Arg^9-BK. In addition, FK 888 and SR 142801, but not SR 48968, were also effective in inhibiting, by $74.6\pm2.3\%$ and $72.7\pm1.9\%$, the influx of mononuclear cells in response to i.t. injection of des-Arg^9-BK ($P<0.01$). Interestingly, only the NK_3 receptor antagonist SR 142801 (10 nmol per site) was able to inhibit significantly des-Arg^9-BK-induced plasma extravasation ($27.4\pm2.2\%$) ($P<0.05$). In contrast, increasing the concentration of the NK_3 antagonist, SR 142801 to 30 nmol per site caused a marked potentiation of the pro-inflammatory response to des-Arg^9-BK with regard to all the parameters studied (results not shown).

The CGRP receptor antagonist, $CGRP_{8-37}$ (1 nmol per site), administered 5 min before pleurisy induction, significantly inhibited ($21.9\pm2.1\%$) des-Arg^9-BK-induced plasma extravasation (Figure 5d) ($P<0.01$), without significantly affecting the migration of mononuclear and neutrophil cells (Figure 5e,f). A combination of the NK_3 antagonist, SR 142801 (10 nmol per site), with the CGRP antagonist, $CGRP_{8-37}$ (1 nmol per site), did not cause any further inhibition of plasma extravasation when compared to that observed with SR 142801 alone (results not shown). The i.t. injection of either FK 888 (1 nmol per site), SR 48968 (20 nmol per site), SR 142801 (10 nmol per site) or $CGRP_{8-37}$ (1 nmol per site) did not result in either an increase of total cell migration or vascular permeability, compared with saline-treated animals ($n=3$, results not shown).

Involvement of nitric oxide, the cyclo-oxygenase pathway and histamine in des-Arg^9-BK-induced pleurisy

The nitric oxide synthase inhibitors L-NOARG (1 pmol per site) or L-NAME (1 pmol per site), when injected alone, had no effect on total cell migration or on plasma extravasation compared with saline-treated groups ($n=3$, results not shown). However, when L-NOARG or L-NAME (1 pmol per site) was administered 30 min beforehand, they almost completely prevented des-Arg^9-BK-induced neutrophil cell migration ($94.8\pm1.8\%$ and $98.5\pm2.1\%$, respectively (Figure 6c,f) ($P<0.01$) and, to a lesser extent, mononuclear cell migration ($45.3\pm2.1\%$ and $66.1\pm1.8\%$) ($P<0.01$) (Figure 6b,e). However, at the same range of doses L-NOARG and L-NAME inhibited total cell migration ($69.8\pm2.0\%$ and $82.1\pm2.2\%$) ($P<0.01$) (results not shown), while L-NAME, but not L-NOARG significantly inhibited ($30.1\pm1.5\%$) des-Arg^9-BK-induced fluid leakage (Figure 6a,d). An i.t. injection of the D-enantiomer, D-NAME (1 pmol per site), had no significant effect on des-Arg^9-BK-induced mouse pleurisy (Figure 6d,e,f).

Treatment of animals with indomethacin (1 mg kg^{-1}, i.p.), administered 1 h before des-Arg^9-BK (30 nmol per site), inhibited the mononuclear cells ($47.3\pm2.1\%$) ($P<0.05$), but, surprisingly, increased the neutrophil migration at 4 h

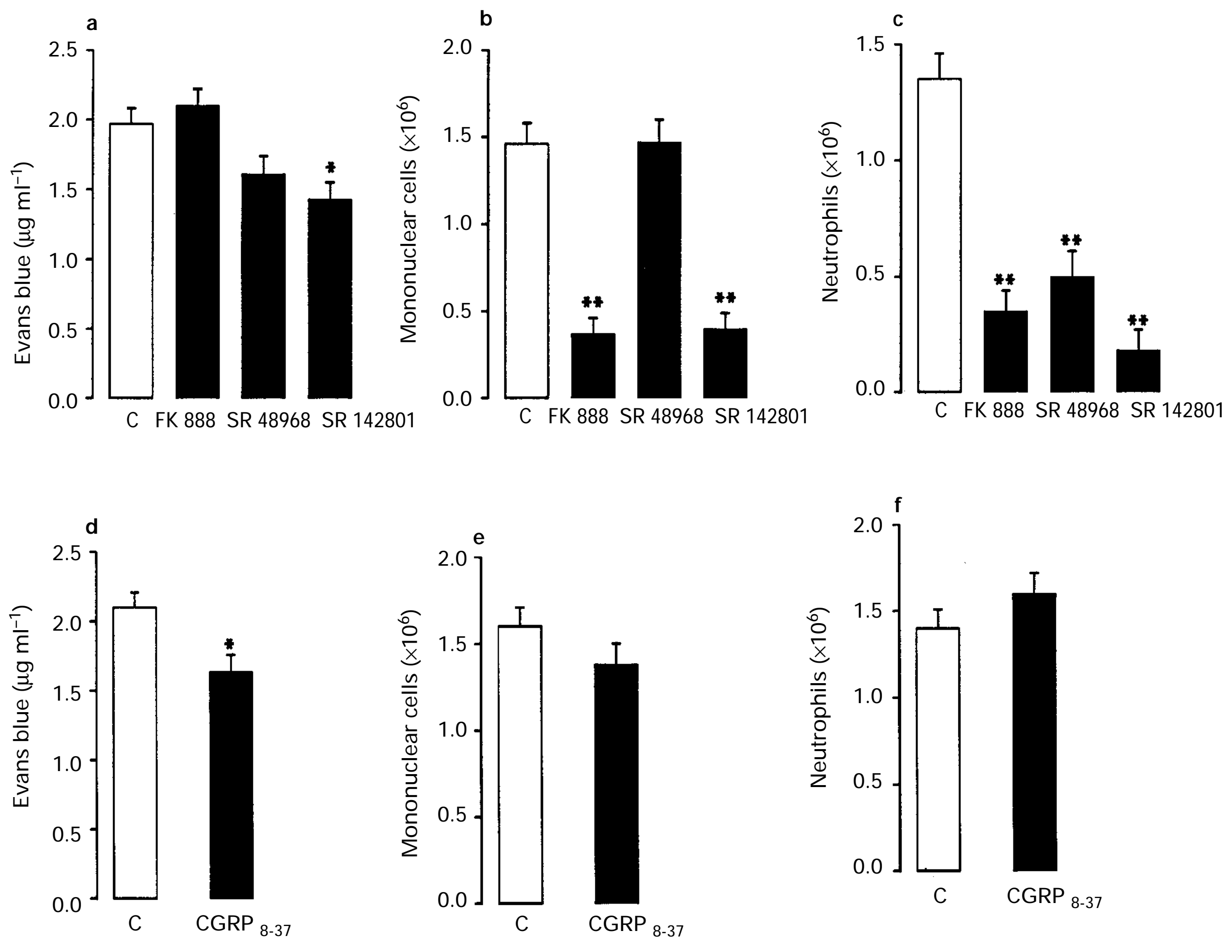

Figure 5 The content of Evans blue extravasation (a,d), the mononuclear cells (b,e) and neutrophils (c,f) induced by i.t. injection of des-Arg^9-BK (30 nmol per site) in the pleural cavity of the mice in the absence (open columns) or presence (solid columns) of the i.t. injection of either (a,b,c) FK 888 (1 nmol per site), SR 48968 (20 nmol per site), SR 142801 (10 nmol per site), or (d,e,f) CGRP$_{8-37}$ (1 nmol per site) all administered 5 min before the i.t. injection of des-Arg^9-BK. Asterisks in each column indicate statistically significant differences compared with control animals. Each column represents the mean of 5 to 6 animals, with s.e.means also shown. *$P<0.05$ and **$P<0.01$.

(35.9±2.5%) ($P<0.01$) (Figure 7b,c). The same treatment of animals with indomethacin did not significantly inhibit plasma extravasation in response to i.t. injection of des-Arg^9-BK (Figure 7a).

The administration of terfenadine (50 mg kg^{-1}, i.p.), 30 min before des-Arg^9-BK (30 nmol per site), did not interfere significantly, with the mouse pleurisy, with the total cell migration or with plasma extravasation caused by i.t. injection of des-Arg^9-BK (control response for total cells of $3.0\times10^6\pm0.11$ cells and exudate of 2.1 ± 0.15 μg ml^{-1} versus $3.2\times10^6\pm0.16$ cells and 2.5 ± 0.15 μg ml^{-1} in the presence of the drug, respectively, $n=5$ in each group) ($P>0.05$).

Effect of treatment of animals with lipopolysaccharide of the Escherichia coli *on des-Arg^9-BK-induced pleurisy and paw oedema*

Pretreatment of the mice, 24 h before an i.t. injection of des-Arg^9-BK, with LPS (10 μg per animal, i.v.) did not result in any significant alteration in the pattern of inflammatory response caused by i.t. injection of this peptide into mouse pleural cavity. The control responses were: $3.2\times10^6\pm0.15$ for total cell influx and 2.5 ± 0.18 μg ml^{-1} for exudation versus $2.9\times10^6\pm0.15$ cells for total cells and 2.3 ± 0.18 μg ml^{-1} for exudation, in animals treated with LPS, respectively, ($n=10$ in each group) ($P>0.05$).

The results in Figure 8 show the paw oedema caused by a subplantar injection of des-Arg^9-BK (30 nmol per paw) in saline and LPS pretreated mice. The subplantar injection of des-Arg^9-BK (50 nmol per paw) in saline pretreated animals caused a partial, but time-dependent paw oedema formation (maximal effect of 22.0 ± 7 μl at 10 min). However, des-Arg^9-BK (30 nmol per paw) caused a greater time-dependent paw oedema in animals that had been treated previously with LPS (maximal effect of 68.0 ± 8 μl at 10 min) ($P<0.01$).

Discussion

The results of the present study, to our knowledge, have demonstrated for the first time that the i.t. injection of the selective B_1 agonists, des-Arg^9-BK and Lys-des-Arg^9-BK, results in a time and dose-dependent and significant inflammatory response characterized by an increase in fluid leakage, associated with an increase in cell migration, mainly neutrophils, and, to a lesser extent, mononuclear cell migration. The inflammatory responses induced by des-Arg^9-BK peaked at 1 h (plasma extravasation) and at 4 h (cell

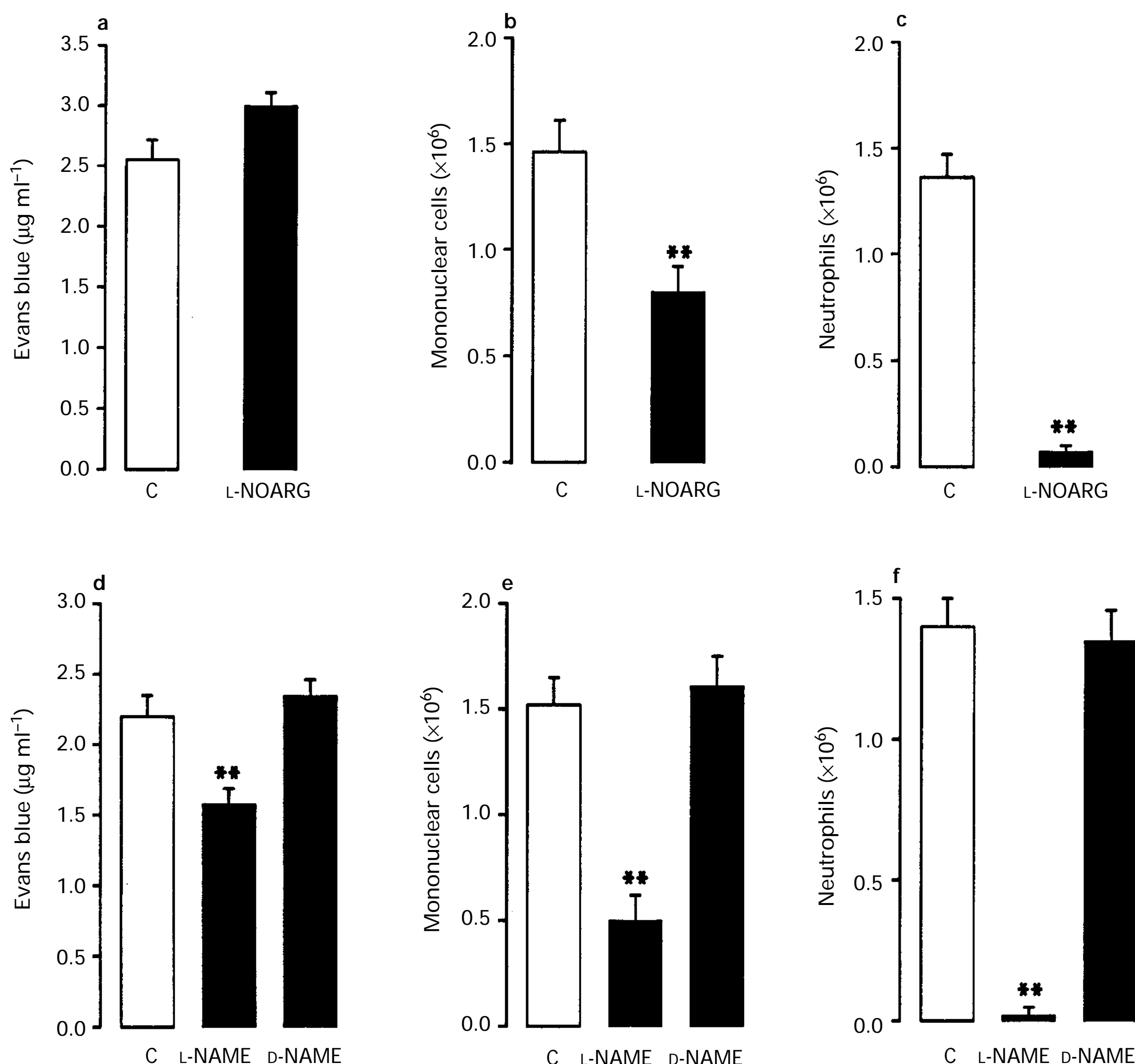

Figure 6 The content of Evans blue extravasation (a,d), the mononuclear cells (b,e) and neutrophils (c,f) induced by des-Arg[9]-BK (30 nmol per site) in the pleural cavity of the mice in the absence (open columns) or presence (solid columns) of the i.t. injection of (a,b,c) L-NOARG, (d,e,f) L-NAME or D-NAME (1 pmol per site) administered 30 min before the i.t. injection of des-Arg[9]-BK. Asterisks in each column indicate the statistically significant differences compared with control animals. Each column represents the mean of 5 to 6 animals, with s.e.means also shown. $*P<0.05$ and $**P<0.01$.

migration) after peptide challenge, and were of long duration (up to 24 h).

The inflammatory responses induced by des-Arg[9]-BK seem likely to be mediated by an effect of this peptide at the B_1 receptor because: (1) the i.t. injection of the selective B_1 receptor antagonists, des-Arg[9][Leu[8]]-BK and mainly des-Arg[9]-NPC 17731, resulted in a consistent attenuation of both the increase in plasma extravasation and in the polymorphonuclear neutrophil migration induced by i.t. injection of des-Arg[9]-BK; and (2) the i.t. injection of the selective and potent B_2 receptor antagonist Hoe 140, at a dose which has been demonstrated to inhibit BK-induced cell migration by more than 70% (Saleh *et al.*, 1997), did not significantly affect des-Arg[9]-BK-induced pleurisy in mice. Interestingly, contrasting with the potency expected by Lys-des-Arg[9]-BK at the B_1 receptor (Regoli & Barabé, 1980; Marceau, 1995), this peptide exhibited essentially the same activity as des-Arg[9]-BK in inducing pleurisy in mice. Similar results have been found by Hess *et al.* (1996) and Pesquero *et al.* (1996), who showed that the cloned mouse B_1 receptor possesses 2 to 3 fold higher selectivity for des-Arg[9]-BK than for Lys-des-Arg[9]-BK. An opposite order of potency has been obtained in rabbit and human cloned B_1 receptors (Menke *et al.*, 1994). Pesquero and colleagues (1996) suggest that such differences may be the consequence of a distinct sequence of amino acids for the B_1 receptors found in these species.

Consistent with our recent observation on the mouse isolated vas deferens, where des-Arg[9]-BK reduced the amplitude of twitch response induced by electrical stimulation through activation of possibly prejunctional constitutive B_1 receptors (Maas *et al.*, 1995) and the inhibition caused by B_1 and B_2 receptor antagonists in formalin-induced nociception in the mouse (Corrêa & Calixto, 1993), the inflammatory response caused by i.t. injection of the B_1 agonist des-Arg[9]-BK in the mouse model of pleurisy seems likely to be mediated by activation of the constitutive B_1 receptor. This view is based on the following observations: (1) the inflammatory responses elicited by des-Arg[9]-BK appeared immediately after the peptide injection (5 min); (2) des-Arg[9]-BK elicited essentially the same inflammatory response in saline-treated mice and in animals that had been pretreated with LPS. In marked contrast, the same treatment of mice with LPS resulted in a

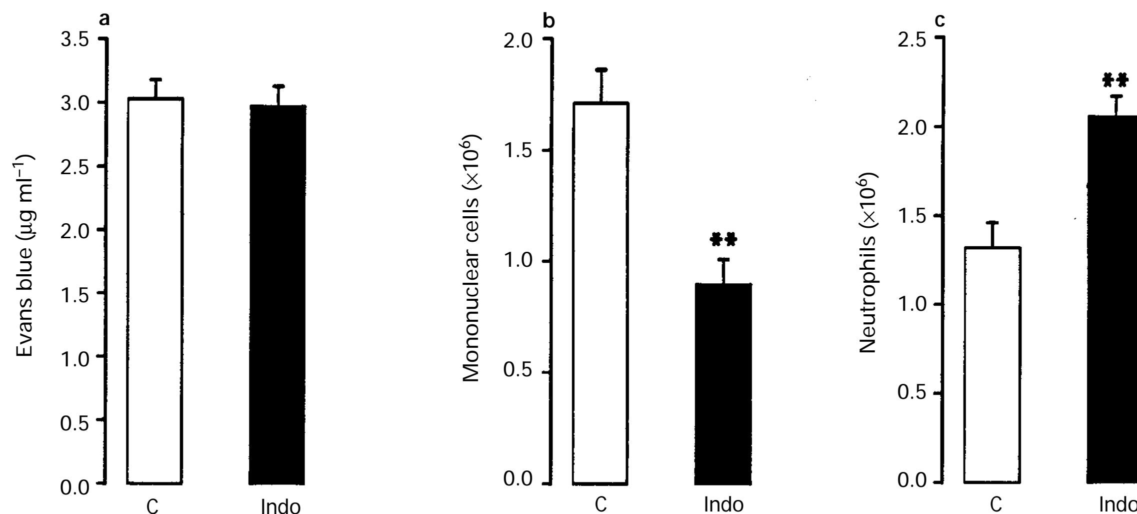

Figure 7 The content of Evans blue extravasation (a), the mononuclear cells (b) and neutrophils (c) induced by i.t. injection of des-Arg^9-BK (30 nmol per site) in the pleural cavity of control mice (C) or in animals treated with indomethacin (Indo; 1 mg kg^{-1}, i.p.) 1 h before the i.t. injection of des-Arg^9-BK. Asterisks in each column indicate the statistically significant differences compared with control animals. Each column represents the mean of 5 to 6 animals, with s.e.means also shown. $^{**}P<0.01$.

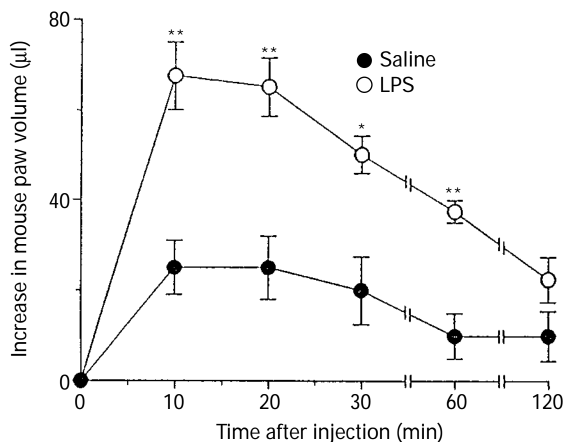

Figure 8 Curves showing the increase in mice paw oedema with time caused by subplantar injection of the selective B_1 receptor agonist, des-Arg^9-BK (50 nmol/paw) in sterile saline or in LPS (10 μg per animal) pretreated-animals. Values represent the differences between volumes (in μl) of vehicle-treated and drug-injected paws. Each point represents the mean of 5 to 6 animals; vertical lines show s.e.mean. Significantly different from control values: $^{**}P<0.01$.

significant enhancement of des-Arg^9-BK-mediated paw oedema when compared with the saline treated group. The same LPS treatment was also found to be sufficient to up-regulate responses mediated by the B_1 agonist des-Arg^9-BK, either in mice (Campos *et al.*, 1995) or in rats (Campos *et al.*, 1996). Recently, Pesquero *et al.* (1996) and Hess *et al.* (1996) have supplied a definitive piece of evidence concerning the presence of constitutive B_1 receptors in the mouse. The authors have cloned the gene which encodes the mouse B_1 receptor, and have shown that the mouse B_1 receptor shares about 68 to 73% of amino acid identity compared with that of rabbit and human B_1 receptors. Interestingly, Pesquero and colleagues (1996) observed a marked up-regulation of B_1 receptors, mainly in heart, lung and liver of mice, after treatment with LPS. The lack of upregulation of des-Arg^9-BK-induced pro-inflammatory effect in the murine model of pleurisy, but not in paw oedema and formalin-induced pain, under the same experimental conditions, is difficult to interpret at the present time. Additional studies are necessary to clarify this point.

A relevant and new finding of the present study was the demonstration that des-Arg^9-BK-mediated pleurisy in mice was largely attenuated by the administration of the selective NK_1 antagonist, FK 888 (Fujii *et al.*, 1992; Murai *et al.*, 1993), NK_2 antagonist SR 48968 (Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992; Kamikawa & Shimo, 1993), and in part the newly-developed NK_3 antagonist SR 142801 (Emonds-Alt *et al.*, 1995), and by the administration of an antagonist of calcitonin gene-related peptide, $CGRP_{8-37}$ (Escott & Brain, 1993). It has been well documented that BK actions on airways are intimately associated with activation of sensory neurones and release of neuropeptides such as substance P, neurokinin A and CGRP (Ichinose & Barnes, 1990; for review see: Geppetti, 1993; Bertrand & Geppetti, 1996; Saleh *et al.*, 1997). Thus, the present results extend these observations and show that the B_1 agonist des-Arg^9-BK-mediated inflammatory response in a murine model of pleurisy involves activation of sensory neurones, which in turn release the neuropeptides. However, there are some important differences in the role of these peptides in des-Arg^9-BK inflammatory response. While NK_1 and NK_3 receptors seem to play an important modulatory role in migration of neutrophil and mononuclear cells, NK_2 receptors appear to participate only in the control of neutrophil influx in response to i.t. injection of des-Arg^9-BK. On the other hand, only the NK_3 receptor subtype appears to be involved in des-Arg^9-BK-induced plasma extravasation.

Several lines of evidence suggest that most of the kinin effects induced by the activation of B_1 and B_2 receptors are mediated indirectly by the release of nitric oxide (NO) or NO-related substance (Sung *et al.*, 1988; Khalil & Helme, 1992; Wiemer & Wirth, 1992; Schlemper & Calixto, 1994; Nakamura *et al.*, 1996). Thus, we also assesssed in the present study whether NO could contribute to the inflammatory response induced by des-Arg^9-BK in the mouse model of pleurisy. I.t. injection of a very low dose of the NO synthase inhibitors (L-NOARG or L-NAME, 1 pmol per site) consistently and significantly inhibited des-Arg^9-BK-mediated lung inflammation. In marked contrast, the inactive D enantiomer (D-NAME 1 pmol) was completely inactive. Such results clearly show that

NO plays an important role in the B_1 selective agonist des-Arg^9-BK-mediated inflammatory response in the murine model of pleurisy. Inhibitors of NO synthase have been demonstrated to prevent neutrophil chemotaxis and degranulation (Belenky *et al.*, 1993; Wyatt *et al.*, 1993). However, in sharp contrast to that found for des-Arg^9-BK-mediated rat paw oedema in animals treated with either LPS (Campos *et al.*, 1996), with BCG (Campos *et al.*, 1997) or after desensitization by repeated injection of a B_2 agonist (Campos *et al.*, 1995), the des-Arg^9-BK-mediated response in the mouse model of pleurisy was surprisingly potentiated by treatment of animals with indomethacin. The reason for such differences is currently unclear, but it could be associated with the different mechanism of action of des-Arg^9-BK on constitutive (present study) compared to that of up-regulated B_1 receptors. Recently, Moraes *et al.* (1996) showed that both aspirin and indomethacin significantly increase the neutrophil infiltration in response to inflammatory aerosol administration of LPS to mice, an effect which was associated to an increase in TNF-α production and suppression of prostaglandin E_2 production. On the other hand, results of the present study show that the treatment of animals with terfenadine, at dose which has been shown to inhibit histamine action (1 μmol per site) (results not shown), did not interfere with the des-Arg^9-BK-mediated inflammatory response, suggesting the lack of involvement of histamine in this response.

It has been shown that the selective B_1 agonist des-Arg^9-BK is able to stimulate the release of some inflammatory mediators, such as prostaglandin I_2 (PGI_2) and PGE_2 (Toda *et al.*, 1987; Cahill *et al.*, 1988; Galizzi *et al.*, 1994), and interleukin-1 (IL-1) (Tyffany & Burch, 1989; Lerner & Modeer, 1991). Also, a synergistic interaction between des-Arg^9-BK and IL-1, BK and several inflammatory mediators, has been demonstrated to occur (Lerner & Modeer, 1991; Bathon *et al.*, 1992; Lerner *et al.*, 1992; Campos & Calixto, 1995; Campos *et al.*, 1996). Furthermore, IL-1 and IL-2, as well as LPS treatment, are able to induce up-regulation of kinin B_1 receptors (Deblois *et al.*, 1988; 1991). Thus, it seems apparent from these and previous data, that the B_1 receptor plays a central regulatory role in the control of chronic inflammatory states. As kinins and tachykinins are involved in the pathophysiology of bronchial function, including in asthma and allergic rhinitis (see for review: Barnes *et al.*, 1988; Barnes, 1992; Hall, 1992; Dray & Perkins, 1993; Bertrand & Geppetti, 1996), the B_1 antagonists could have important therapeutic relevance in the management of such diseases.

In conclusion, we have demonstrated, for the first time, that the inflammatory response induced by i.t. injection of des-Arg^9-BK in a murine model of pleurisy is mediated by stimulation of constitutive B_1 receptors. These responses are largely mediated by the release of neuropeptides such as substance P, CGRP and also by NO, but do not involve the participation of cyclo-oxygenase metabolites derived from arachidonic acid pathway and histamine. Therefore, these results suggest that B_1 selective receptor antagonists might provide therapeutic benefit in the treatment of inflammatory and allergic processes.

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