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# Differential effect of L-NAME and S-methyl-isothiourea on leukocyte emigration in carrageenin-soaked sponge implants in rat

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**1** The role of nitric oxide (NO) in leukocyte (polymorphonuclear cells, monocytes and lymphocytes) emigration was studied in a model of carrageenin-sponge implants in rats.

**2** The subcutaneous implantation of 1% (w/v) of $\lambda$-carrageenin-soaked sponges elicited an inflammatory response that was characterized by a time-related increase in leukocyte infiltration in the sponges and increased levels of nitrite in the exudate. Total leukocyte infiltration and nitrite production were maximal at 24 h and decreased after 48 and 96 h. The mononuclear cell influx was maximal at 48 h (21% of the total leukocytes). Therefore, this time point was used in the successive experiments.

**3** Polymorphonuclear cell (PMN) and lymphocyte infiltration in the sponges significantly increased when rats were treated with the non-specific NO-synthase (NOS) inhibitor, $N^G$-nitro-L-arginine methylester (L-NAME) (1 mg ml$^{-1}$ in drinking water *ad libitum*). Monocyte emigration was not affected by L-NAME treatment. The nitrite levels in the exudate of L-NAME-treated rats were significantly reduced. The concomitant ingestion of L-arginine (30 mg ml$^{-1}$) resulted in a reversion of the L-NAME effect, while D-arginine (30 mg ml$^{-1}$) had no effect, indicating the involvement of the L-arginine: NO pathway.

**4** Administration of L-NAME resulted also in an increased release of tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and prostacyclin (measured as the stable metabolite, 6-keto-$PGF_{1\alpha}$). L-NAME had no effect on monocyte chemoattractant protein-1 (MCP-1) release in the exudate.

**5** Since L-NAME may have effects on the local blood flow, phenylephrine (0.034 mg ml$^{-2}$ in drinking water) was used as it has an effect on the local blood flow similar to L-NAME. Phenylephrine had no effect on either leukocyte emigration, or on nitrite, TNF-$\alpha$, prostacyclin or MCP-1 accumulation in the exudate.

**6** In contrast, the more selective iNOS inhibitor S-methyl-isothiourea (SMT) (10 $\mu$g ml$^{-1}$ in drinking water) significantly reduced PMNs and lymphocyte influx in the sponge, having no effect on monocyte influx. Moreover, SMT decreased nitrite production in the exudate to a comparable extent as L-NAME.

**7** Administration of SMT significantly reduced MCP-1 release in the exudate, without an effect on TNF-$\alpha$ or prostacyclin production. Moreover SMT did not produce any changes in local blood flow.

**8** Our results show that a different outcome of the inflammatory process can be obtained depending on the types of NOS inhibitor used.

**Keywords:** Nitric oxide; S-methyl-isothiourea; L-NAME; leukocyte emigration; PMNs; lymphocytes; carrageenin; sponge implants; TNF-$\alpha$; prostacyclin; MCP-1; phenylephrine

## Introduction

The first and critical step in the cascade of an inflammatory response is the leukocyte-endothelium interaction followed by leukocyte extravasation into the inflamed area: within a few minutes polymorphonuclear leukocytes (PMNs) arrive followed by monocytes and lymphocytes (Osborn, 1990). The leukocyte infiltration is a multistep process mediated by different adhesion molecules on both the surface of leukocytes and on microvascular endothelial cells (Osborn, 1990; Butcher, 1991). The infiltration of leukocytes occurs in response to a gradient of chemotactic factors (Baggiolini *et al*., 1994).

At the site of an inflammatory reaction, the injured vascular endothelial cells and the emigrated leukocytes release a large number of chemical mediators which modulate and maintain the inflammation. NO is generated enzymatically from the amino acid L-arginine by a family of enzymes called NO synthases (NOS). At least two types of NOS have so far been identified: the constitutive NOS (cNOS), which is $Ca^{2+}$/calmodulin-dependent, has been found in endothelial cells (endothelial NOS or eNOS) and in the brain (neuronal NOS or nNOS). The inducible NOS (iNOS), which is $Ca^{2+}$/calmodulin-independent, has been found in activated macrophages, neutrophils, endothelial cells and smooth muscle cells (for review see Morris & Billiar, 1994). Both enzymes are inhibited *in vivo* and *in vitro* by several L-arginine analogues including $N^G$-nitro-L-arginine methyl ester (L-NAME) (Moncada *et al*., 1991). Recently, a novel class of more potent and selective iNOS inhibitors, including S-methyl-isothiourea (SMT), have been described (Southan *et al*., 1995).

NO has been shown to have both pro- and anti-inflammatory actions: NO generated by the constitutive isoform of NOS in the vascular endothelium is involved in the regulation of the blood pressure and organ blood flow distribution and inhibits *in vitro* neutrophil adhesion to the endothelium (McCall *et al*., 1988) and *in vivo* leukocyte adhesion to cat (Kubes *et al*., 1991) and rat (Arndt *et al*., 1993) postcapillary venules. In contrast, NO produced by the inducible isoform increases carrageenin-

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induced plasma extravasion in rat skin and rat paw-oedema induced by dextran and carrageenin (Ialenti *et al*, 1992). Granuloma formation by carrageenin (Iuvone *et al*., 1994) and adjuvant arthritis in rats (Ialenti *et al*., 1993; McCartney *et al*., 1993) are also increased by endogenous NO production.

The aim of the present study was to clarify the possible inflammatory or inhibitory role of NO on leukocyte emigration in a model of carrageenin-soaked sponge implants in rats. We studied the effect of L-NAME and the more selective iNOS inhibitor, SMT, to investigate the contribution of cNOS and iNOS activation in the development of this type of inflammation. In addition, we investigated whether the leukocyte emigration was correlated to an increase of some inflammatory mediators: tumour necrosis factor-α (TNF-α), prostacyclin and monocyte chemoattractant protein-1 (MCP-1).

## Methods

### *Sponge implantation*

Sponges were implanted as described by Iuvone *et al*. (1994). Briefly two polyether sponges ($0.5 \times 1.5 \times 2.0$ cm) were implanted subcutaneously on the back of male Wistar rats weighing 200–250 g. Sponges and surgery tools were sterilized by autoclaving. 0.5 ml of 1% λ-carrageenin in pyrogen free saline or saline was injected into each sponge; in some experiments 0.2 mM Evan's blue was coinjected in the right sponge to determine the dilution of the dye as result of plasma leakage. Various treatments were started one day before implantation: the rats received L-NAME (1 mg $ml^{-1}$), D- or L-arginine (30 mg $ml^{-1}$), L-NAME plus L-arginine, SMT (1 and 10 μg $ml^{-1}$) or phenylephrine (0.034 mg $ml^{-1}$) dissolved in tap water. The water intake (*ad libitum*) of rats was $40 \pm 10$ ml $day^{-1}$ and was not significantly different between the treatments. The rats were killed 48 h after sponge implantation.

In preliminary time-course experiments the effect of carrageenin on leukocyte emigration and nitrite production was studied after 24, 48, 96 h of saline- and carrageenin-soaked sponge implantations.

### *Collection of cells and exudate*

The sponges were removed, centrifuged at 400 *g* for 15 min; the extravasated cells plus fluid in the sponge were collected in plastic sterile 15 ml tubes containing heparin 5 u $ml^{-1}$ and indomethacin 30 mM. The exudate volume in the sponge was measured and stored at −20°C for the assays. The exudate volume, measuring $1.0 \pm 0.2$ ml, did not change over the time course and treatments. The cell pellet was resuspended in 1 ml of saline and total cell amount was counted by Coulter Counter (LTD Harpenden Herts, U.K.). Differential counts were performed on cytospin preparations of cells stained by Auto-Hemacolor (Merck) from Hematek slide stainer.

### *Nitrite assay*

Nitrite accumulation in the exudate was determined spectrophotometrically according to Schmidt *et al*. (1988). Therefore, protein precipitation was performed after addition of 30% zinc sulphate and centrifugation at 350 *g* for 20 min; 200 μl of the sample was added to 40 μl of 1:1 (v/v) diluted HCl 6.5 M and sulphanilic acid 37.5 mM; 20 μl of N-(1-naphtyl)-ethylenediamine 12.5 mM was added after 10 min and the absorbance at 450 nm was measured 30 min thereafter.

### *Prostacyclin assay*

The release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) the stable metabolite of prostacyclin, was measured by radioimmunoassay (RIA) according to Laekeman *et al*. (1986). Tris buffer, standard or samples, 0.5% bovine gammaglobulin, antibody (1/3000 diluted) and [$^3$H]-6-keto-$PGF_{1\alpha}$ (final activity of 8000 d.p.m./tube) in a final volume of 0.7 ml were mixed and incubated overnight at 4°C. Then proteins were precipitated with an equal volume of polyethylene glycol (25% in distilled water) at 0°C and immediately centrifuged (1800 *g*, 1 h at 0°C); 1 ml of the supernatant plus 3 ml of distilled water was mixed with 9 ml UltimaGold (Packard) and radioactivity was counted. With every batch of samples a standard curve (4 to 2000 pg of 6-keto-$PGF_{1\alpha}$) was included. Values for unknown samples were calculated according to a four parametric model described by Dudley *et al*. (1985).

### *TNF-α assay*

The level of TNF-α in the exudate was quantified by its dose-dependent cytotoxic effect on WEHI-164 cells. Briefly, 50 μl WEHI-164 ($65 \times 10^4$ cells $ml^{-1}$) in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal calf serum were plated on 96-well plates and allowed to adhere at 37°C in 5% $CO_2$/95% air for 2 h. Thereafter 25 μl of actinomycin-D (10 mg $ml^{-1}$) and 50 μl aliquots of 1:4 v/v serial dilution of each sample were added and then the cells were incubated for 24 h. The cell viability was assessed by reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) to MTT-formazan. After 3 h incubation period with 25 μl of MTT (5 mg $ml^{-1}$) cells were lysed and the dark blue crystals of formazan salt were solubilized with 100 μl of a solution containing 50% N,N-dimethylformamide, 20% (w:v) SDS (sodium dodecylsulphate) at pH 4.5. Overnight incubation was followed by measuring the optical density of the samples at 620 nm. TNF-α bioactivity was calculated from a standard curve of human recombinant TNF-α ranging from 31.25 to 4000 u $ml^{-1}$. Rabbit anti-murine TNF-α antiserum (Genzyme) which cross-reacts with rat TNF-α was used in order to assess the specificity of TNF-α-dependent cytotoxicity (Garrelds *et al*, 1993).

### *MCP-1 assay*

The amount of MCP-1 in the exudate was measured by an ELISA kit (Cytoscreen rat MCP-1, Biosource). Briefly, 100 μl of samples were added to a 96-well plate coated with a specific antibody for rat MCP-1 followed by the addition of a biotinylated secondary antibody and incubation for 1 h 30 min. After removal of excess secondary antibody, streptavidin-peroxydase was added and incubated for another 1 h 30 min. The chromogen tetramethylbenzidine was used as substrate which was acted upon by the bound enzyme to produce colour. The intensity of this coloured product was directly proportional to the concentration of MCP-1 present in the samples. Absorbance was measured at 450 nm and the concentration of unknown samples was related to the MCP-1 standard curve ranging from 39 to 2500 pg $ml^{-1}$.

### *Evan's blue experiments*

The absorbance of 100 μl of exudate from the sponges receiving Evan's blue (0.2 mM) plus λ-carrageenin was read at 620 nm with a 96-micro well reader (Titertex Multiskan MCC/340). The dilution factor was measured as absorbance of the exudate versus the absorbance of Evan's blue dye at the start of experiment.

### *Statistical analysis*

Data are expressed as total amounts of nitrite, prostacyclin, TNF-α, MCP-1 or cells per sponge. All values are expressed as the mean ± s.e.mean of *n* observations, where *n* is the number of sponges studied. Statistical comparisons were made by one way-ANOVA followed by a Bonferroni's test for multiple comparisons, or by Mann Whitney-U non-parametric test for unpaired samples.

### Materials

All compounds obtained were of the highest quality from Sigma Chemical Company, unless otherwise stated. All the material for cell culture were purchased from Gibco BRL.

## Results

### Time-course experiments

Carrageenin (1% w/v) induced a significant increase of leukocyte extravasion into the sponge after 24 and 48 h (versus saline). The relative amount of mononuclear cells (monocytes and lymphocytes) was much higher after 48 than after 24 h: $21\pm1.2\%$ versus $10\pm0.9\%$ ($n=12$). In parallel with the leukocyte infiltration, local generation of NO (measured as nitrite) was increased after 24 and 48 h, and slowly decreased after 96 h (Table 1).

**Table 1** Time-course for the accumulation of the total number of leukocytes (total cells) and nitrite in the exudate of carrageenin-soaked versus saline-soaked sponge implants in rats

| | *Total cells* ($1\times10^6$) | | *Nitrite* (nmol) | |
|---|---|---|---|---|
| *Time* (h) | *Saline* | *Carrageenin* | *Saline* | *Carrageenin* |
| 24 | $1.5\pm0.5$ | $14.4\pm1.1$ | $4.5\pm0.05$ | $24.1\pm0.5$ |
| 48 | $0.81\pm0.01$ | $8.3\pm1.0$ | $4.5\pm0.05$ | $21.4\pm1.1$ |
| 96 | $0.24\pm0.06$ | $1.0\pm0.7$ | $4.5\pm0.05$ | $13.9\pm0.9$ |

The data show the mean of 12–18 sponges$\pm$s.e.mean.

For all further experiments the 48 h time point was chosen to evaluate the involvement of NO in the process of leukocyte influx into the sponge and the release of TNF-$\alpha$, prostacyclin and MCP-1.

### Effect of L-NAME and L-arginine on leukocyte infiltration and nitrite production

Treatment with L-NAME (1 mg ml$^{-1}$) resulted in a significant increase in the total number of infiltrated leukocytes ($15.5\pm1.9\times10^6$ total cells; $P<0.01$) as compared to the control rats ($71\pm0.9\times10^6$ total cells) (Figure 1a). The number of PMNs and lymphocytes was significantly increased ($13.2\pm1.8\times10^6$ PMNs, $P<0.01$ and $1.1\pm0.2\times10^6$ lymphocytes, $P<0.01$) as compared to control ($5.9\pm0.8\times10^6$ PMNs and $0.34\pm0.06\times10^6$ lymphocytes), whereas monocyte influx was not significantly changed ($1.2\pm0.2\times10^6$ monocytes in control rats and $1.8\pm0.3\times10^6$ monocytes in L-NAME-treated rats). L-Arginine (30 mg ml$^{-1}$) did not affect leukocyte influx ($7.0\pm1.2\times10^6$ total cells; $5.6\pm1.0\times10^6$ PMNs; $1.1\pm0.2\times10^6$ monocytes; $0.5\pm0.1\times10^6$ lymphocytes) (Figure 1a). Treatment of the rats with L-NAME reduced nitrite levels in the exudate ($15.1\pm0.9$ nmol; $P<0.05$) whereas L-arginine increased the nitrite levels ($28.6\pm1.4$ nmol; $P<0.05$) as compared to controls ($23.1\pm1.3$ nmol) (Figure 1b).

To investigate whether we could reverse the effect of L-NAME, L- or D-arginine (30 mg ml$^{-1}$) was given in combination of L-NAME (1 mg ml$^{-1}$) (Figure 2a). The effect of L-NAME was significantly reversed by concomitant ingestion of L-arginine ($11.4\pm2.5\times10^6$ total cells; $P<0.05$ versus

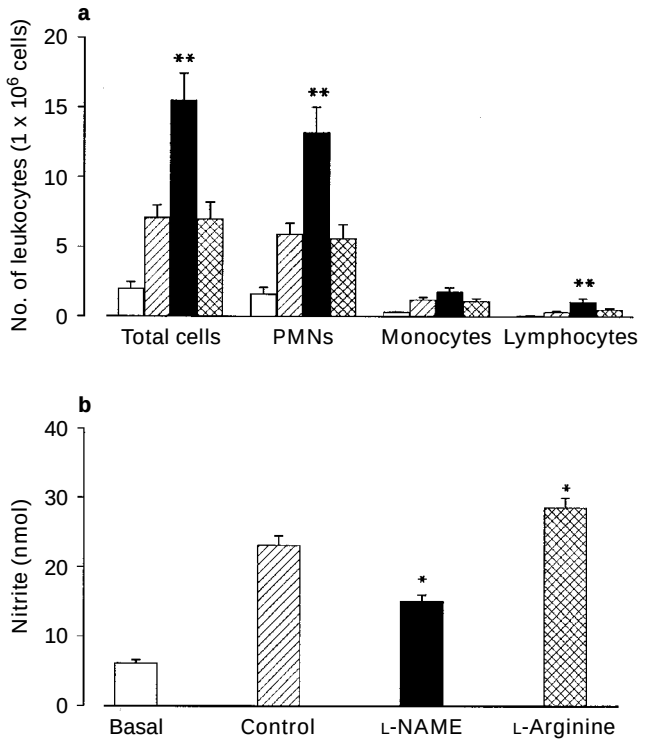


**Figure 1** (a) Effect of L-NAME (1 mg ml$^{-1}$) and L-arginine (30 mg ml$^{-1}$) on leukocyte emigration (saline = open columns; control = hatched columns; L-NAME = solid columns; L-arginine = cross-hatched columns) in rat sponge implants after 48 h. Saline represents rats receiving saline-soaked sponge implants and drinking tap water. Control, L-NAME and L-arginine represent rats receiving carrageenin-soaked sponge implants and drinking, respectively, tap water, L-NAME and L-arginine. Values are the means of $n=24-26$ sponges$\pm$s.e.mean. Statistical significance, $^{**}P<0.01$ vs control. (b) Effect of L-NAME (1 mg ml$^{-1}$) and L-arginine (30 mg ml$^{-1}$) on nitrite production in the exudate of rat sponge implants after 48 h. Columns represent the mean of $n=20-22$ sponges$\pm$s.e.mean. Statistical significance, $^{*}P<0.05$ vs control.

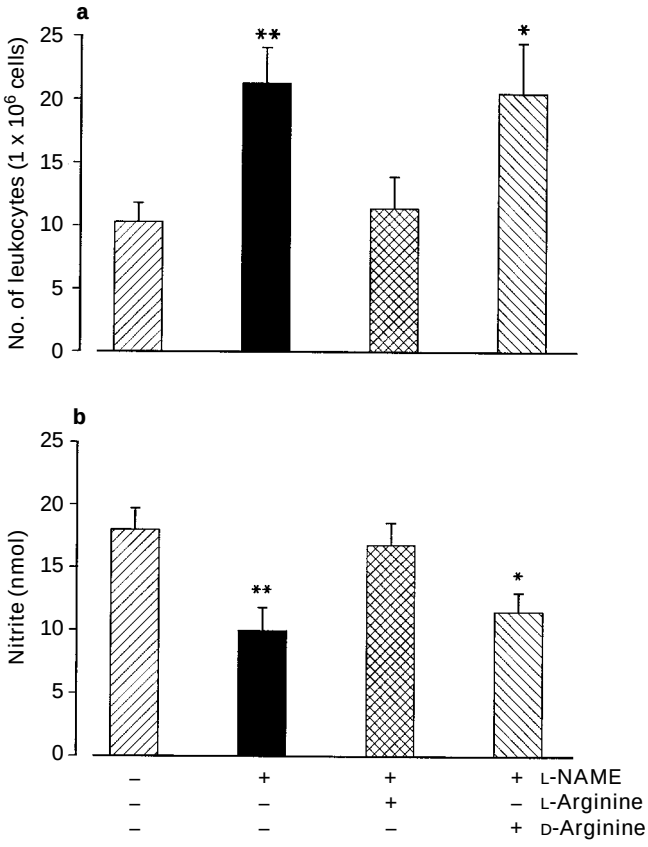


**Figure 2** Effect of L-NAME (1 mg ml$^{-1}$), L-arginine (30 mg ml$^{-1}$), D-arginine (30 mg ml$^{-1}$) on: (a) total leukocyte migration; (b) nmol of nitrite production in carrageenin-soaked sponge implants in rats after 48 h. Animals received, respectively, tap water (control), L-NAME, L-NAME plus L-arginine, L-NAME plus D-arginine in drinking water. Columns represent the mean of $n=20-22$ sponges$\pm$s.e.mean. Statistical significance, $^{*}P<0.05$, $^{**}P<0.01$ vs control.

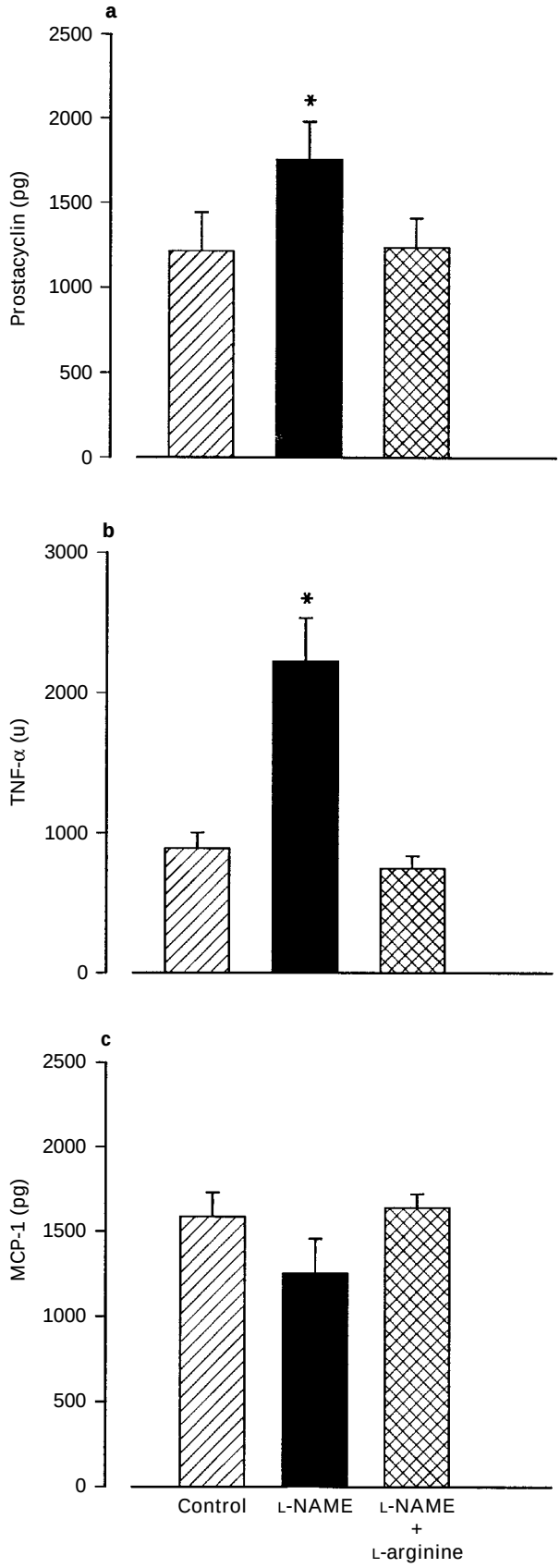


**Figure 3** Effect of L-NAME (1 mg ml$^{-1}$) and L-NAME plus L-arginine (30 mg ml$^{-1}$) on: (a) prostacyclin (b) TNF-α (c) MCP-1 release in the exudate of carrageenin-soaked sponge implants in rats after 48 h. Animals received, respectively, tap water (control), L-NAME and L-NAME plus L-arginine in drinking water. Columns represent the mean ± s.e.mean of $n = 22-24$ sponges. Statistical significance, $*P < 0.05$ vs control.

L-NAME, NS, versus control) but not by D-arginine ($20.5 \pm 4.0 \times 10^6$ total cells) (Figure 2a). The simultaneous ingestion of L-NAME plus L-arginine also completely restored the nitrite values from 10.0 ± 1.8 nmol in L-NAME-treated rats to 16.8 ± 1.6 nmol in L-NAME plus L-arginine-treated rats as compared to control values (18.0 ± 1.7 nmol) (Figure 2b).

### *Effect of L-NAME and L-arginine on prostacyclin, TNF-α and MCP-1 production*

Inhibition of NOS by L-NAME elicited a significant increase of 6-keto $PGF_{1\alpha}$ in the exudate (1758 ± 221 pg, $P < 0.05$) as compared to control (1215 ± 228 pg) (Figure 3a). This increase was reversed when L-NAME was combined with L-arginine (1235 ± 174 pg). In the exudate of saline-soaked implanted rats the levels of 6-keto $PGF_{1\alpha}$ were not detectable.

The levels of TNF-α in the exudate of control rats (889 ± 114 u) were significantly increased by L-NAME (2232 ± 503 u; $P < 0.05$) and reversed by simultaneous ingestion of L-NAME plus L-arginine (749 ± 89 u) (Figure 3b). TNF-α levels in saline-soaked sponge exudates were not detectable.

In the exudate of saline-soaked sponges MCP-1 measured 335 ± 65 pg. The levels of MCP-1 in control animals (1589 ± 143 pg) were not significantly affected by L-NAME (1259 ± 81 pg) or by the simultaneous ingestion of L-NAME plus L-arginine (1642 ± 200 pg) (Figure 3c).

### *Experiments with SMT*

We next examined the effect of the more selective iNOS inhibitor SMT in this model. In contrast to L-NAME, SMT (10 μg ml$^{-1}$) decreased PMNs and lymphocyte influx in the

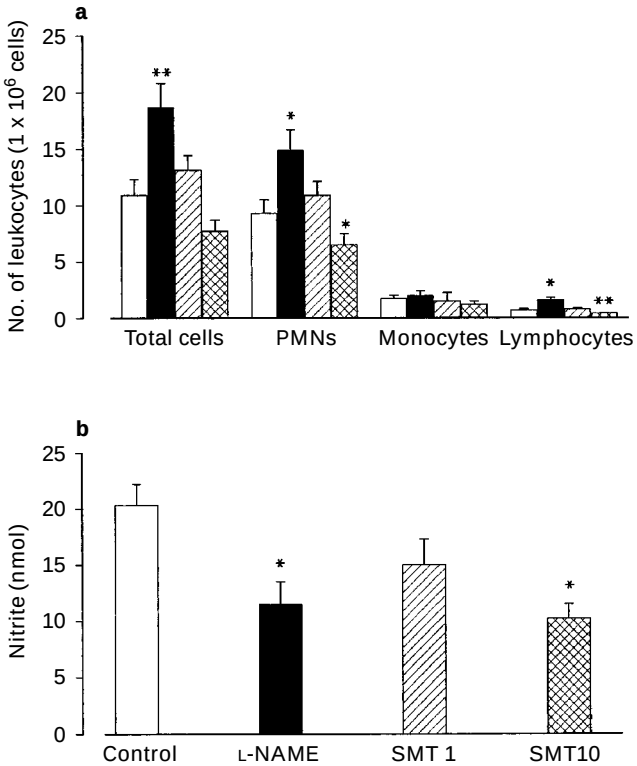


**Figure 4** Comparison of the effect of S-methyl-isothiourea (SMT) (1 and 10 μg ml$^{-1}$) with L-NAME (1 mg ml$^{-1}$) on: (a) leukocyte migration (control = open columns; L-NAME = solid columns; SMT 1 μg = hatched columns; SMT 10 μg = cross-hatched columns), (b) nitrite production, in the exudate of carrageenin-soaked sponge implants in rats at 48 h. Control, L-NAME and SMT represent rats drinking, respectively, tap water, L-NAME and SMT. Values are the mean of $n = 20-26$ sponges ± s.e.mean. Statistical significance, $*P < 0.05$, $**P < 0.01$ vs control.

sponge ($6.5 \pm 1.0 \times 10^6$ PMNs; $0.4 \pm 0.04 \times 10^6$ lymphocytes; $P < 0.01$) as compared to the controls ($9.3 \pm 1.2 \times 10^6$ PMNs; $0.7 \pm 0.1 \times 10^6$ lymphocytes) (Figure 4a). Monocyte influx was not significantly decreased by SMT ($1.2 \pm 0.3 \times 10^6$ cells in SMT-treated rats versus $1.7 \pm 0.3 \times 10^6$ cells in control rats). SMT at a lower concentration (1 $\mu$g ml$^{-1}$) did not modulate leukocyte emigration into the sponge.

SMT (10 $\mu$g ml$^{-1}$) decreased nitrite production in the exudate ($10.2 \pm 1.3$ nmol; $P < 0.05$) to a comparable extent as L-NAME ($11.5 \pm 2.0$ nmol; $P < 0.05$), whereas SMT (1 $\mu$g ml$^{-1}$) had no significant effect ($15.0 \pm 2.3$ nmol) (Figure 4b).

In contrast to the effect of L-NAME on prostacyclin and TNF-$\alpha$ release, SMT did not affect the release of either prostacyclin ($1191 \pm 93$ pg in SMT-treated rats; $1052 \pm 56$ pg in control rats) or TNF-$\alpha$ ($1136 \pm 88$ u in SMT-treated rats; $1200 \pm 92$ u in control rats) (Figure 5a and b), whereas MCP-1 levels were significantly reduced by SMT ($749 \pm 127$ pg in SMT-treated rats; $1165 \pm 113$ pg in control rats; $P < 0.05$) (Figure 5c).

### *Measurement of plasma leakage with Evan's blue*

It is well known that both acute and chronic administration of L-NAME *in vitro* causes elevated mean arterial blood pressure and local vasoconstriction (Gardiner *et al.*, 1990; Bryant *et al.*, 1995). In our experiments we determined the dilution of Evan's blue to measure plasma exudation which may be a result of changes in local blood flow. The initial concentration of Evan's blue injected into the sponge (0.2 mM) was almost 20 times diluted in the control exudate ($9.7 \pm 0.9$ $\mu$M) whereas in L-NAME-treated animals Evan's blue was significantly less diluted ($20.3 \pm 2.6$ $\mu$M) (Figure 6a). In contrast, SMT had no effect on plasma exudation ($10.1 \pm 1.3$ $\mu$M).

To exclude the possibility that some of the effects of L-NAME were due to an effect on plasma exudation related to local vasoconstriction, we investigated the effect of phenylephrine (0.034 mg ml$^{-1}$) (Vasquez *et al.*, 1994; Hale & Kloner, 1994), a potent vasoconstrictor agent. Phenylephrine reduced Evan's blue dilution to a comparable extent as L-NAME (Figure 6b) but, in contrast to L-NAME, phenylephrine did not affect the total and differential cell count or the production of prostacyclin, TNF-$\alpha$ and MCP-1 (Table 2).

## Discussion

Plasma exudation and leukocyte emigration into the extravascular tissue are hallmarks of inflammation supported by the production of several inflammatory mediators. The role of NO in this complex inflammatory process is contradictory. Both cNOS and iNOS may be involved during plasma exudation and leukocyte infiltration into the inflamed tissue. Therefore, we investigated the role of NO in a model of carrageenin-soaked sponge implants in rats by use of two NOS inhibitors: a non-selective NOS inhibitor, L-NAME, and the more selective iNOS inhibitor SMT (Southan *et al.*, 1995). Both inhibitors exhibited differential effects on leukocyte emigration and plasma exudation in our inflammatory model, although they inhibited the nitrite production to the same extent. Our results demonstrated that combined inhibition of both cNOS and iNOS by L-NAME increases leukocyte infiltration and this effect can be reversed by the addition of L-arginine. In our model L-arginine alone failed to decrease leukocyte infiltration although it was able to increase nitrite production. This apparent discrepancy may be due to the dose of L-arginine used, in that it was able to increase nitrite production but not able to inhibit leukocyte emigration. On the other hand it has already been shown that the NO-donor, S-nitroso-N-acetyl penicillamine, significantly decreases leukocyte rolling and adherence to the endothelium (Gauthier *et al.*, 1994). In contrast to L-NAME, inhibition of iNOS by SMT inhibited leukocyte infiltration into the sponge.

Several inflammatory mediators, such as arachidonic acid metabolites are released in carrageenin-induced inflammation and may be regulated via NO. Our study showed that inhibi-

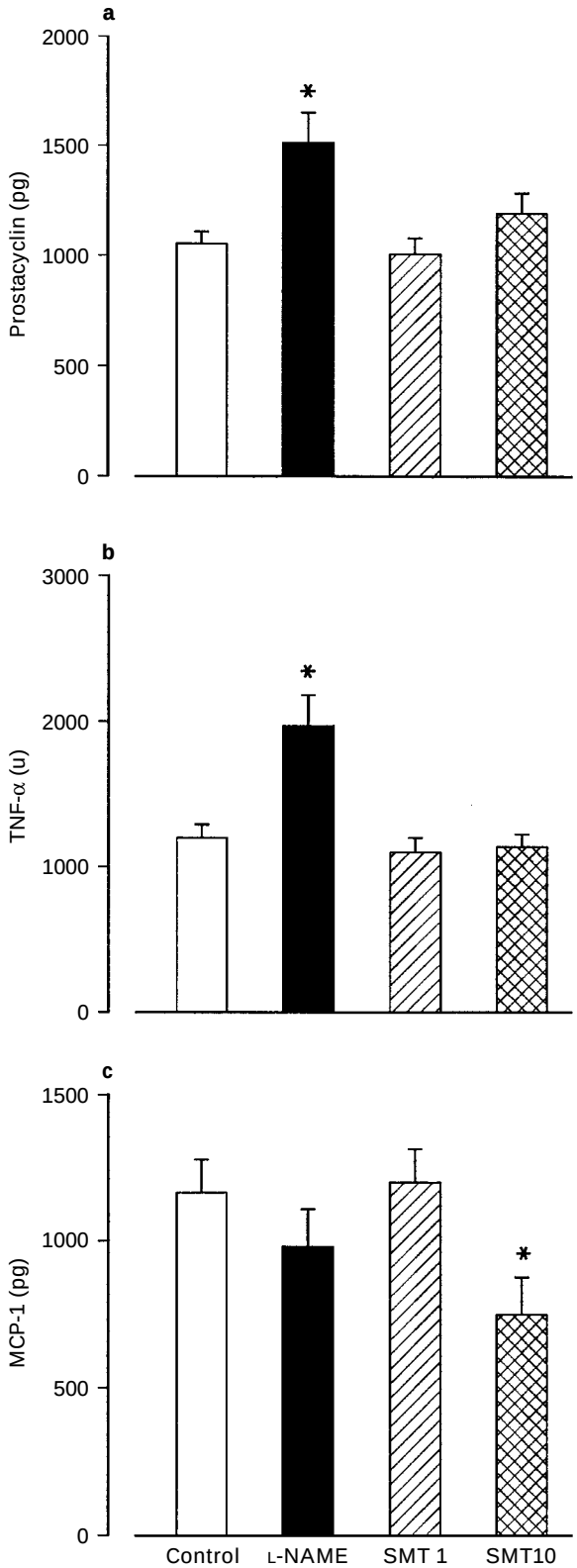


**Figure 5** Effect of L-NAME (1 mg ml$^{-1}$) and S-methyl-isothiourea (SMT, 1 and 10 $\mu$g ml$^{-1}$) on: (a) prostacyclin; (b) TNF-$\alpha$; (c) MCP-1 release in the exudate of carrageenin-soaked sponge implants in rats after 48 h. Animals received, respectively, tap water (control), L-NAME and SMT in drinking water. Columns represent the mean $\pm$ s.e.mean of $n = 22-24$ sponges. Statistical significance, $^*P < 0.05$ vs control.

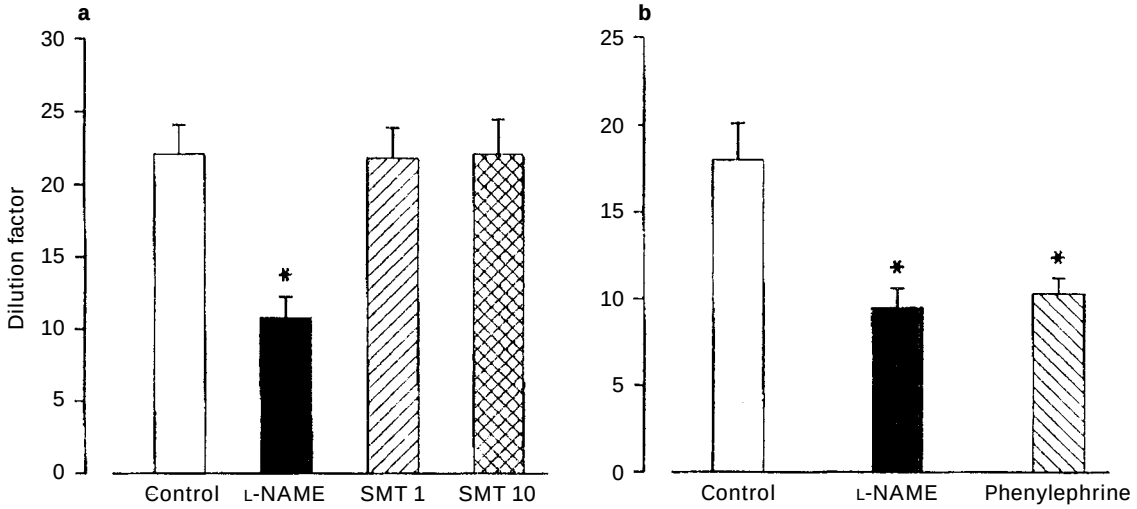


**Figure 6** Plasma exudation in carrageenin-soaked sponge implants in rats after 48 h. Effect of: (a) L-NAME (1 mg $ml^{-1}$) and S-methyl-isothiourea (SMT, 1 and 10 $\mu g$ $ml^{-1}$); (b) L-NAME (1 mg $ml^{-1}$) and phenylephrine (0.034 mg $ml^{-1}$). The plasma leakage is expressed as a dilution factor comparing the absorbance of each exudate to the absorbance of carrageenin plus Evan's blue injected at the start of the experiment. Columns represent mean ± s.e.mean of $n = 16-18$ sponges. Statistical differences, $*P < 0.05$.

**Table 2** Comparison of the effects of phenylephrine (0.034 mg $ml^{-1}$) and L-NAME (1 mg $ml^{-1}$) on leukocyte infiltration and release of nitrite, prostacyclin, TNF-α and MCP-1 in carrageenin-soaked sponge implants at 48 h

| | *Treatments* | | |
|---|---|---|---|
| | *Control* | *L-NAME* (1 mg $ml^{-1}$) | *Phenylephrine* (0.034 mg $ml^{-1}$) |
| Total cells ($1 \times 10^6$) | 11.0 ± 2.3 | 26.9 ± 3.8** | 13.0 ± 2.3 |
| PMNs | 8.2 ± 1.9 | 21.1 ± 4.4** | 12.0 ± 1.5 |
| Monocytes | 2.0 ± 0.8 | 4.0 ± 0.5 | 2.3 ± 0.6 |
| Lymphocytes | 0.6 ± 0.2 | 1.8 ± 0.3 | 0.6 ± 0.07 |
| Nitrite (nmol) | 16.5 ± 2.3 | 9.9 ± 0.1** | 15.7 ± 3.1 |
| Prostacyclin (pg) | 1288 ± 43 | 1700 ± 149** | 1110 ± 77 |
| TNF-α (u) | 920 ± 110 | 2005 ± 200** | 899 ± 90 |
| MCP-1 (pg) | 1332 ± 45 | 1130 ± 138 | 1360 ± 90 |

The values represent the mean ± s.e.mean of 12 sponges. Statistical significance, $**P < 0.01$ vs control.

tion of NO production by L-NAME resulted in an increased release of prostacyclin in the exudate. Accumulating evidence indicates that many interactions occur between the L-arginine: NO and cyclo-oxygenase pathways (for review see Di Rosa *et al*., 1996), although the results of these studies appear controversial. It has been found that NO may increase (Salvemini *et al*., 1993; 1995; Sautebin *et al*., 1995) or decrease (Doni *et al*., 1988; Keen *et al*., 1990; Stadler *et al*., 1993) prostaglandin biosynthesis. These discrepancies may be the result of differences in the experimental conditions, or in the different models of inflammation used or in the administration regime of the drugs. The effect of L-NAME on prostacyclin release can explain the elevated number of infiltrated leukocytes, in our model. Indeed, a synergism has been described between extravascular prostaglandins and other inflammatory mediators, like TNF, to increase vascular permeability and leukocyte influx (Issekutz, 1981; Williams, 1983; Rampart *et al*., 1989).

In our experiments the inhibition of NO release by L-NAME was also associated with an increase of TNF-α in the exudate. TNF-α has been shown to induce PMN infiltration *in vivo* and to induce the expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 on endothelial cells (Springer, 1990).

Various factors related to the cyclo-oxygenase and NO pathway can regulate the synthesis of TNF-α *in vitro* and *in vivo*. Inhibition of cyclo-oxygenase significantly enhances the serum level of TNF-α in murine (Pettipher & Wimberly, 1994) and human (Martich *et al*., 1991) endotoxic shock. Recently, it has been found that NOS-inhibition increases lipopolysaccharide (LPS)-induced TNF-α production in RAW 264.7 and J774 macrophages (Eigler *et al*., 1995; Iuvone *et al*., 1996) and in LPS-treated rats (Iuvone *et al*., 1996). Therefore, our results suggest that the increased leukocyte emigration observed after L-NAME treatment results from an elevation of TNF-α levels in the exudate. Nevertheless, we cannot exclude the possibility that the increased levels of both prostacyclin and TNF-α are related to an increased number of infiltrated leukocytes. In fact it is well known that L-NAME increases leukocyte emigration through mast cell degranulation and release of superoxide anions, as described by Kubes *et al*. (1993). In turn, the increased number of infiltration leukocytes may be responsible for the increase in prostacyclin and TNF-α in our model.

In contrast to L-NAME, SMT inhibited leukocyte influx and had no effect on prostacyclin and TNF-α release in the exudate. Thusfar, there is no evidence that the more specific iNOS inhibitors have any effect on mast cell degranulation and this may explain the different effects of SMT compared to L-NAME.

Penetration of the vascular endothelium by blood leukocytes occurs in response to a gradient of chemotactic factors released from the cells of the vasculature. MCP-1 is a well studied monocyte chemotactic factor, which exerts potent and specific chemoattractant activity for both monocytes and T-lymphocytes (Carr *et al*., 1994; Taub *et al*., 1995). Moreover monocytes and, to a lesser extent, also granulocytes are found to secrete MCP-1 in response to cytokines, viruses, bacterial endotoxins and mitogenes (Wuyts *et al*., 1994). Recently, it has been shown that inhibition of cNOS by $N^G$-nitro-L-arginine induced MCP-1 in human endothelial cells *in vitro* (Zeiher *et al*., 1995), suggesting that NO modulates MCP-1 expression and activity *in vitro*. However, in our *in vivo* experiments, NO inhibition by L-NAME did not affect MCP-1 release. In contrast to L-NAME, SMT decreased MCP-1 release in the exudate, which is probably the underlying mechanism of the decrease in lymphocyte emigration by SMT in our study. These apparently contrasting data may be explained by considering the different experimental condition (*in vitro* and *in vivo* model), the cell-type studied (endothelial cells and infiltrating leukocytes) or the NOS isoform predominantly involved in different experimental models.

It is well known that both acute and chronic administration of L-NAME increase mean arterial blood pressure (Gardiner *et*

*al*., 1990; Bryant *et al*., 1995). Changes in blood pressure and local vasoconstriction may finally result in less plasma exudation according to the 'two mediator-principle', supporting a synergism between vasodilatation on the arteriolar side and increased vascular permeability on the venular side (Williams, 1977). In our experiments, we found that L-NAME produced less plasma exudation, probably due to local vasoconstriction at the sponge site. In contrast, according to Szabo *et al*. (1994) SMT does not change local blood flow. Therefore, we questioned whether the increased amount of some mediators in the exudate of the L-NAME-treated animals could be due to such vasoconstrictor effect. However, although phenylephrine produced a local vasoconstriction to a similar extent as L-NAME, it failed to increase the exudate levels of nitrite, prostacyclin, TNF-$\alpha$ and MCP-1.

Our results clearly demonstrate that, depending on the type of NOS inhibitor used, a different effect on the inflammatory process can be obtained in the same *in vivo* model of inflammation.

We suggest that the multidirectional action of NOS inhibitors may increase the understanding of the dual activity of NO in the inflammatory process. Furthermore, by inhibiting leukocyte infiltration, the more specific iNOS inhibitors may be useful in the treatment of certain inflammatory reactions which are associated with an enhanced formation of NO due to the induction of iNOS.

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