Br. J. Pharmacol. (1993), **110**, 43–49 © Macmillan Press Ltd, 1993

Stimulation of angiogenesis by substance P and interleukin-1 in the rat and its inhibition by NK_1 or interleukin-1 receptor antagonists

[1]Tai-Ping D. Fan, De-En Hu, *Steven Guard, †G. Austin Gresham & *Keith J. Watling

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ; *Parke-Davis Neuroscience Research Centre, Addenbrooke's Hospital Site, Hills Road, Cambridge CB2 2QB and †Department of Histopathology, Addenbrooke's Hospital, Cambridge CB2 2QQ

1 Daily administration of 1 nmol substance P or 3 pmol recombinant human interleukin-1 alpha (IL-1α) caused intense neovascularization in a rat sponge model of angiogenesis. Lower doses of substance P (10 pmol) or IL-1α (0.3 pmol) were ineffective when given alone. When combined at these low doses, substance P and IL-1α interacted to produce an enhanced neovascular response.

2 By use of selective tachykinin NK_1, NK_2 and NK_3 receptor agonists, ([Sar^9,Met(O$_2$)^{11}]substance P, [β-Ala^8]neurokinin A(4-10), Succ-[Asp^6,MePhe^8]substance P(6-11) (senktide), respectively), it was established that the activation of NK_1 receptors is most likely to mediate the angiogenic response to substance P in this model.

3 The angiogenic activity of substance P and IL-1α (10 pmol and 0.3 pmol day^{-1}, respectively) was abolished by co-administration of (i) the selective peptide NK_1 receptor antagonist, L-668,169 (1 nmol day^{-1}), (ii) the selective non-peptide NK_1 receptor antagonists, RP 67580 and (±)-CP-96,345 (both at 1 nmol day^{-1}) or (iii) the IL-1 receptor antagonist, IL-1ra, (50 μg day^{-1}). In contrast, the selective NK_2 receptor antagonist, L-659,874 (1 nmol day^{-1}) was ineffective.

4 The angiogenic action of substance P and IL-1α was resistant to modification by mepyramine (1 nmol day^{-1}) and/or cimetidine (10 nmol day^{-1}), indomethacin (7 nmol day^{-1}) or the platelet-activating factor (PAF) antagonist, WEB-2086 (22 nmol day^{-1}), indicating that histamine, prostaglandins and PAF are not likely to be involved in this neovascular response.

5 The inhibition of the substance P/IL-1 angiogenic response by selective NK_1 receptor antagonists or by an IL-1 receptor antagonist demonstrates that angiosuppression can be achieved by blocking the activity of angiogenic factors at the receptor level.

Keywords: Angiogenesis; angiosuppression; substance P; tachykinin receptors; NK_1 receptor antagonists; interleukin-1; interleukin-1 receptor antagonist; rheumatoid arthritis

Introduction

Angiogenesis, the formation and growth of new capillary blood vessels, is an important process in many physiological conditions such as embryonic development and wound healing. However, defects in the controlling mechanism of angiogenesis often result in pathological conditions e.g. rheumatoid synovial hypertrophy, atherosclerosis, proliferative retinopathy and solid tumours. It is now widely recognised that both growth factors and inhibitors are involved in the regulation of vascular growth (see Folkman & Klagsbrun, 1987; Klagsbrun & D'Amore, 1991; Moses & Langer, 1991; Fan & Brem, 1992, for reviews).

The neuropeptides, substance P and calcitonin gene-related peptide (CGRP), are important mediators of inflammation. In addition to their vasodilator activity (Brain & Williams, 1989; Payan, 1989), they are mitogenic for cells derived from the vasculature and connective tissues. For example, substance P stimulates the proliferation of arterial smooth muscle cells, skin fibroblasts, synovial cells and endothelial cells (Nilsson *et al.*, 1985; Lotz *et al.*, 1987; Ziche *et al.*, 1990) and CGRP is mitogenic for endothelial cells (Haegerstrand *et al.*, 1990). These *in vitro* data raise the possibility that substance P and other neuropeptides may be involved in angiogenesis. In support of this hypothesis, substance P has been shown to stimulate neovascularization in rabbit cornea (Ziche *et al.*, 1990).

The interactions between cytokines and inflammatory mediators have been investigated by many groups in the last few years. For example, Lotz *et al.* (1988) demonstrated that substance P increases the production of inflammatory cytokines, including interleukin 1 alpha (IL-1α) by cultured monocytes, while Kimball & Fisher (1988) reported substance P to potentiate IL-1-induced BALB/3T3 fibroblast proliferation. *In vivo*, interactions between IL-1 and CGRP have been shown to induce inflammatory oedema (Buckley *et al.*, 1991). Recently, we presented preliminary evidence that in addition to substance P, other peptides such as CGRP and vasoactive intestinal polypeptide (VIP) also stimulate angiogenesis in a rat sponge model (Fan & Hu, 1991; Hu & Fan, 1991). Furthermore, some of these peptides interact with IL-1α to modulate the neovascular response. Thus, neuropeptides may contribute to the aberrant neovascularization often associated with chronic inflammatory diseases.

In view of the fact that at least three receptor types, termed NK_1, NK_2 and NK_3, are believed to mediate the biological effects of tachykinins (see Guard & Watson, 1991, for review), we have characterized the tachykinin receptor(s) involved in the substance P/IL-1α response, using selective tachykinin receptor agonists and antagonists. In addition, we have examined the effects of indomethacin, a platelet-activating factor (PAF) antagonist and histamine receptor antagonists in order to examine whether the synergistic interaction of substance P and IL-1α is mediated by prostaglandins, PAF or histamine.

[1] Author for correspondence.

Methods

The sponge implant model

Circular sponge discs (1.2 cm diameter) were prepared from a sheet of 5 mm thick polyether foam. A 1.2 cm segment of polythene tubing (1.4 mm internal diameter) was secured to the interior of each sponge disc by means of 5/0 silk sutures so that every sponge disc possessed a central cannula. Before implantation, sponge discs were soaked in 70% ethanol for 2–3 h and then rinsed in sterile phosphate buffered saline (PBS). After squeezing the sponges in a 20 ml syringe to remove excess PBS, they were sterilised by overnight ultraviolet light irradiation.

Implantation of sponge discs was performed with aseptic techniques. Hypnorm (0.5 ml kg^{-1}; 0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone) was used to induce neuroleptanalgesia in male Wistar rats weighing 180–200 g. After the dorsal side had been shaved and wiped with 70% ethanol, a 1 cm dorsal, midline, vertical skin incision was made approximately 4 cm caudal to the occipital ridge. Using a pair of curved scissors, two subcutaneous air-pockets were prepared, one anterior and the other posterior to the incision. Two needle punctures (5 cm apart) were made on top of the pockets. A sterile sponge disc was then inserted into each air-pocket, with the free end of its cannula being exteriorised through the needle puncture. To immobilise the sponge implant, the base of each cannula was sutured to the rat skin. Finally, the skin incision was sutured with two interrupted 5/0 silk stitches, and the cannula was plugged with a sterile polythene stopper so as to prevent overt infection and evaporation of ^{133}Xe-saline during blood flow measurements. The stopper was changed every day and if infection of the implants became apparent, the animals were excluded from the experiments. To prevent the rats from tampering with the cannulae, they were housed individually in plastic cages. Animals were provided with a normal diet and water.

Neovascularisation was assessed as a function of blood flow through the implants over a period of 14 days, by a ^{133}Xe clearance technique (Andrade *et al.*, 1987) and confirmed histologically. Briefly, animals were anaesthetized with Hypnorm as before and 10 µl ^{133}Xe in sterile PBS was injected into the sponges through the cannulae. The washout of radioactivity from the implants was monitored with a gamma scintillation detector. The 6 min ^{133}Xe clearance value was calculated as follows:

$$\%\ ^{133}\text{Xe clearance} = \frac{\text{initial radioactivity} - \text{residual radioactivity at 6 min}}{\text{initial radioactivity}} \times 100\%$$

The validity of this method has recently been established (Fan *et al.*, 1992a,b; Hu *et al.*, 1992; Hu & Fan, 1993). First, we carried out parallel studies of ^{133}Xe clearance and ^{113}Sn microsphere accumulation during sponge-induced angiogenesis. The latter technique enabled us to measure absolute blood flow in the sponges. Second, the ^{133}Xe clearance data have been correlated with the amount of haemoglobin in the sponges. The results from these two studies indicate that measurements of relative blood flow changes in sponge implants by the ^{133}Xe clearance method provide a simple and rapid means to assess new blood vessel formation, when confirmed by histological studies.

Test substances dissolved in PBS were administered through attached cannulae into sponges in a total volume of 50 µl daily, starting on day 1 after implantation, until day 10. To exclude the possible acute effects of the test substances (dilatation or constriction) on the microvasculature, they were given 16–24 h prior to the ^{133}Xe measurements.

Histology

For histology, animals were killed by cervical dislocation and sponges dissected out, carefully removing the cannulae and any adherent fat. The samples were then bisected and fixed in formal saline at 4°C for 1 h. Sections (10 µm) were prepared from paraffin-embedded blocks and stained with haematoxylin and eosin (H&E) or a specific endothelial cell marker *Bandeiraea simplicifolia* lectin I, isolection B_4. The specimens were analysed and recorded on Ektachrome ASA 64T film.

Radioimmunoassay for 6-keto-$PGF_{1\alpha}$

On day 14 after implantation, animals were killed by cervical dislocation and sponges dissected out. Each sponge was placed immediately in 1 ml PBS containing 10 µg ml^{-1} BW755C at 4°C to inhibit further arachidonate metabolism. Sponges were minced in this solution and then centrifuged at 4000 r.p.m. to remove the sponge, cells and debris. The supernatant (sponge fluid) was stored at – 20°C until assay of sponge 6-keto-$PGF_{1\alpha}$ content by a specific radioimmunoassay as described by Fan & Lewis (1984). Unlabelled 6-keto-$PGF_{1\alpha}$, over the concentration range 0.15–80 ng ml^{-1} in 0.1 M tricine buffered saline (TBS) pH 8.0 containing 0.1% gelatin and 0.9% NaCl, was used as standard. Standard or sample (100 µl) was incubated at 4°C for at least 2 h with 100 µl [^{3}H]-6-keto-$PGF_{1\alpha}$ (50 µCi ml^{-1}) and 100 µl antiserum to 6-keto-$PGF_{1\alpha}$ diluted 1/3,000 in TBS. Incubation was terminated by addition of 1.4 ml ammonium calcium sulphate suspension (65% saturated $(NH_4)_2SO_4$, pH 8.0, containing 2.5% $CaSO_4 \cdot 2H_2O$) followed by centrifugation at 2,500 r.p.m. for 10 min. The supernatant was removed by aspiration and the pellet resuspended in 600 µl distilled water before addition of 1.4 ml Instagel scintillant (Packard). After thorough mixing, the gel was allowed to set at 4°C before counting for 1 min in a Packard Tri-Carb 300 scintillation counter. The absolute amount of radioactivity in the gel was counted, the percentage binding calculated and sample values estimated from a standard curve.

Materials

Substance P and [D-Pro^2,D-Phe^7,D-Trp^9]SP were purchased from Peninsula Laboratories, UK. [Sar^9,Met(O$_2$)^{11}]substance P (Drapeau *et al.*, 1987), [β-Ala^8]neurokinin A(4-10) (Rovero *et al.*, 1989) succ-[Asp^6,MePhe^8]substance P(6-11) (senktide)(Wormser *et al.*, 1986), L-668,169 (cyclo[Gln-D-Trp-(NMe)-Phe(R)-Gly(ANC-2·Leu-Met]$_2$) and L-659,874 (Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2; Williams *et al.*, 1988) were purchased from Cambridge Research Biochemicals, UK. Racemic (±)-CP-96,345 (a mixture of the two enantiomers

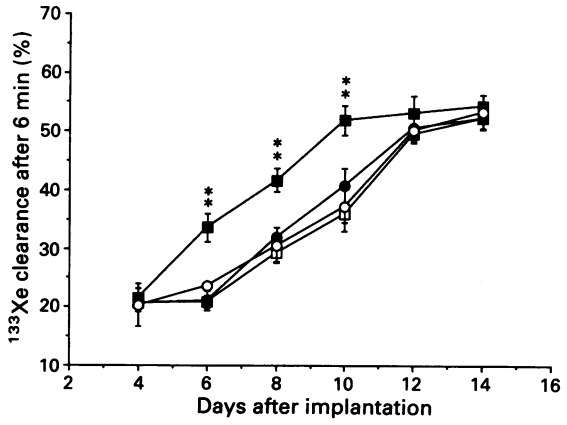

Figure 1 Effect of substance P and interleukin-1α (IL-1α) on sponge-induced angiogenesis. Symbols represent sponges treated daily with PBS alone (□), 10 pmol substance P (○), 0.3 pmol IL-1α (●) or a combination of 10 pmol substance P and 0.3 pmol IL-1α (■); see Methods for details. Each point represents mean data ± s.e.mean from 8–10 animals. $^{**}P < 0.01$ (substance P and IL-1α vs PBS control).

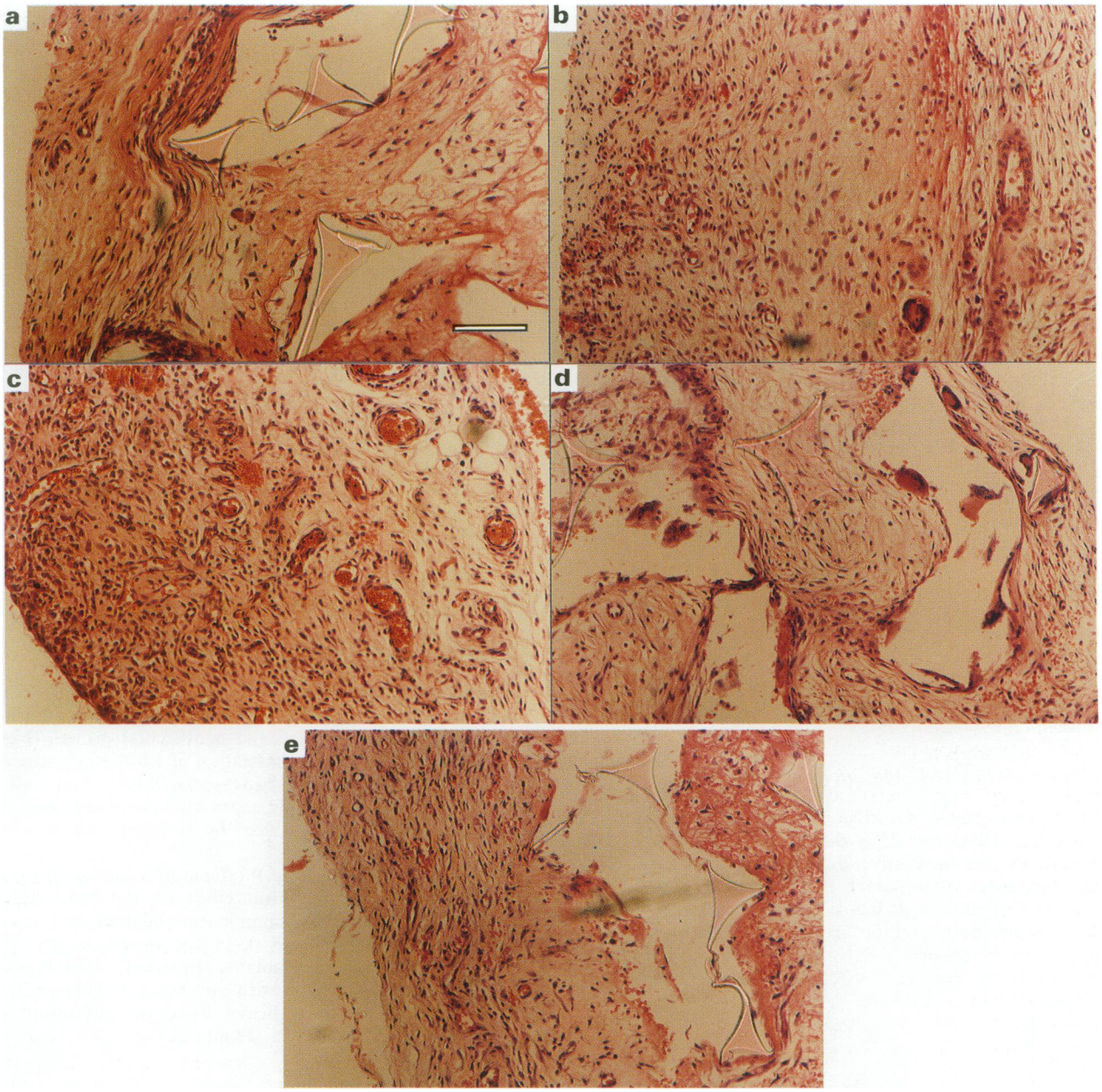

Figure 2 Histological sections of 8 day old sponges illustrating (i) the intense neovascularization induced by substance P alone (1 nmol day^{-1}) or substance P/interleukin-1α (IL-1α) (10 pmol/0.3 pmol day^{-1}), and (ii) the angiosuppressive effect of RP 67580 (1,000 pmol day^{-1}) or IL-1ra (50 μg day^{-1}). All sections were H&E stained and photographed at × 200 magnification. Bar = 100 μm. (a) Sponge treated with PBS; (b) sponge treated with substance P alone; (c) sponge treated with substance P/IL-1α; (d) sponge treated with substance P/IL-1α plus RP 67580; (e) sponge treated with substance P/IL-1α plus IL-1ra.

[(2S,3S) and (2**R**,3**R**)] of the non-peptide NK_1 receptor antagonist (*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl]-methyl]-1-azabicyclo [2.2.2] octan-3-amine; Snider *et al.*, 1991) and RP 67580 ((3a**R**,7a**R**)-7,7,-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one; Garret *et al.*, 1991) were obtained from the Parke-Davis Neuroscience Research Centre, Cambridge. Recombinant human IL-1α, recombinant human IL-1 receptor antagonist (IL-1ra; Hannum *et al.*, 1990), recombinant human basic fibroblast growth factor (bFGF), 6-keto-$PGF_{1\alpha}$ antiserum and the PAF receptor antagonist WEB-2086 (3-[4-(2-chlorphenyl)-9-methyl-6H-thieno [3,2-f] [1,2,4] triazolo-[4,3-a] [1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone; Casals-Stenzel *et al.*, 1987) were gifts from Dr J. Saklatvala of Strangeways Research Laboratory, Cambridge, Dr R.C. Thompson of Synergen Inc., Colorado, U.S.A., Dr M. Presta, Department of Biomedical Sciences and Biotechnology, University of Brescia, Italy, Dr J. Salmon of Wellcome Research Laboratories, Kent, U.K. and Dr C. Meade of Boehringer Ingelheim, Germany, respectively.

^{133}Xenon injection (10 mCi in 3 ml saline) and [^{3}H]-6-keto-$PGF_{1\alpha}$ (50 mCi ml^{-1}) were obtained from Amersham International plc, UK. Other materials or reagents were purchased from the following companies: polyether foam sheet (R.E. Carpenter & Co., Suffolk, UK); polythene tubings (Portex Ltd., UK); Hypnorm (Janssen Pharmaceuticals, UK); indomethacin, mepyramine and cimetidine (Sigma Chemical Co., UK); specific endothelial cell marker *Bandeiraea simplicifolia* lectin I, isolectin B_4 ($BSL\text{-}B_4$, Vector Laboratories Ltd., Peterborough, UK). Indomethacin, and WEB 2086 were prepared in PBS (calcium and magnesium free, pH 7.4) from a stock solution of 10 mg ml^{-1} in ethanol. All other drugs were made up in PBS daily and sterilized by membrane filtration (0.45 μm) before use.

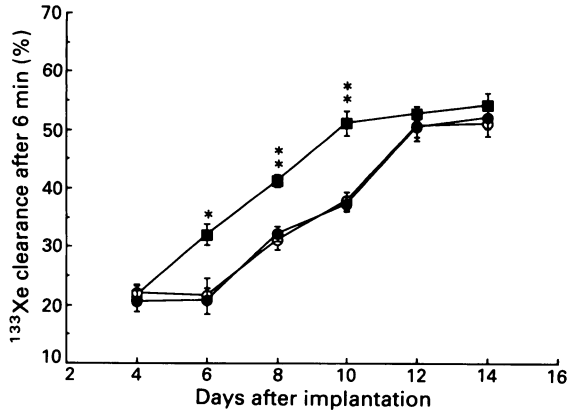

Figure 3 Inhibition of the angiogenic effect of substance P/interleukin-1α (IL-1α) by the substance P antagonist, [D-Pro^2, D-Phe^7, D-Trp^9]substance P. Symbols represent sponges treated daily with substance P/IL-1α (■), substance P/IL-1α plus 100 pmol (●) or 1,000 pmol (○) of the antagonist; see Methods for details. Each point represents mean data ± s.e.mean from 8–10 animals. $*P<0.05$; $**P<0.01$ (substance P/IL-1α plus 1,000 pmol [D-Pro^2, D-Phe^7, D-Trp^9]substance P vs substance P/IL-1α).

Statistical analysis

Statistical analysis of results was performed by a Student's *t* test.

Results

Effects of substance P and interleukin-1α

As shown in Figure 1, the ^{133}Xe clearance in control sponges was between 19% and 22% during the first 6 days after implantation due to passive diffusion of the radioisotope from the sponges. After day 6 the clearance increased such that by day 10 it was 36.0 ± 1.5%, and by day 14 it had reached a maximal level of 52.3 ± 1.7%, which was approaching the clearance obtained in normal rat skin (65–72%).

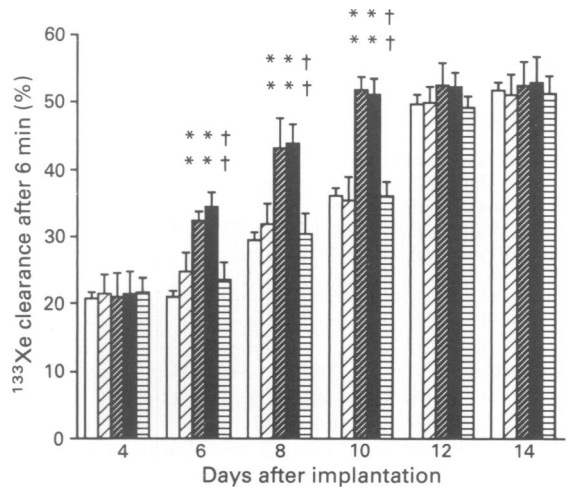

Figure 4 Angiogenic activity of the selective NK_1-receptor agonist [Sar^9,Met(O$_2$)^{11}]SP and its antagonism by the selective NK_1-receptor antagonist, L-668,169. Columns represent sponges treated daily with PBS alone (□), 10 pmol [Sar^9,Met(O$_2$)^{11}]SP (▨), 1,000 pmol [Sar^9,Met(O$_2$)^{11}]SP (▨), 10 pmol [Sar^9,Met(O$_2$)^{11}]SP plus 0.3 pmol interleukin-1α (IL-1α) (■) and 10 pmol [Sar^9,Met(O$_2$)^{11}]SP plus 0.3 pmol IL-1α plus 1,000 pmol L-668,169 (▤). Note that 1,000 pmol L-668,169 alone produced no effect; see Methods for details. Each point represents mean data ± s.e.mean from 6–8 animals. $**P<0.01$ (1,000 pmol [Sar^9,Met(O$_2$)^{11}]SP vs PBS alone: $††P<0.01$ [Sar^9,Met(O$_2$)^{11}]SP/IL-1α vs PBS alone; [Sar^9,Met(O$_2$)^{11}]SP/IL-1α vs [Sar^9,Met(O$_2$)^{11}]SP/IL-1α plus L-668,169).

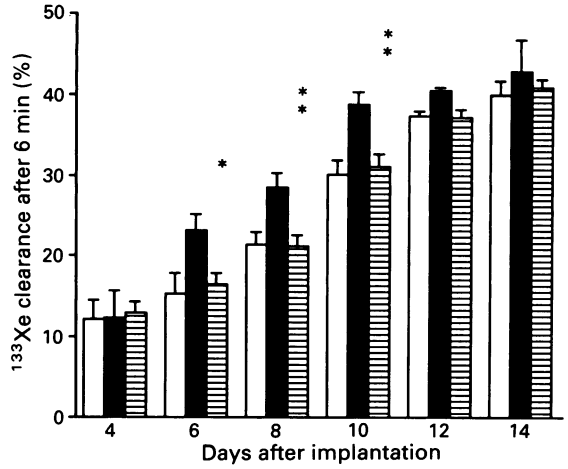

Figure 5 Inhibition of substance P/interleukin-1α (IL-1α)-induced angiogenesis by the non-peptide NK_1 receptor antagonist, RP 67580. Columns represent sponges treated daily with PBS (□), 10 pmol substance P plus 0.3 pmol IL-1α (■) and 10 pmol substance P plus 0.3 pmol IL-1α plus 1,000 pmol RP 67580 (▤). Note that 1,000 pmol RP 67580 alone produced no effect. See Methods for details. Each point represents mean data ± s.e.mean from 6 animals. $*P<0.05$; $**P<0.01$ (substance P/IL-1α plus RP 67580 vs substance P/IL-1α).

Histological studies showed that 8-day-old sponges treated with PBS were encapsulated by connective tissue, but with only little tissue infiltration and neovascular growth (Figure 2a). In contrast, daily administration of 1,000 pmol substance P elicited a substantial neovascularization and cellular infiltration (Figure 2b). This neovascular response was indistinguishable from that produced by daily injection of 3 pmol IL-1α (data not shown).

Lower doses of substance P (10 pmol) or IL-1α (0.3 pmol) alone produced no significant effect on the basal neovascularization. However, the combination of these subthreshold doses of substance P and IL-1α led to accelerated ^{133}Xe clearance by the sponge implants (Figure 1). Histologically, 8-day-old sponges treated with substance P (10 pmol) and IL-1α (0.3 pmol) showed heavy leucocyte infiltration and extensive neovascularisation (Figure 2c).

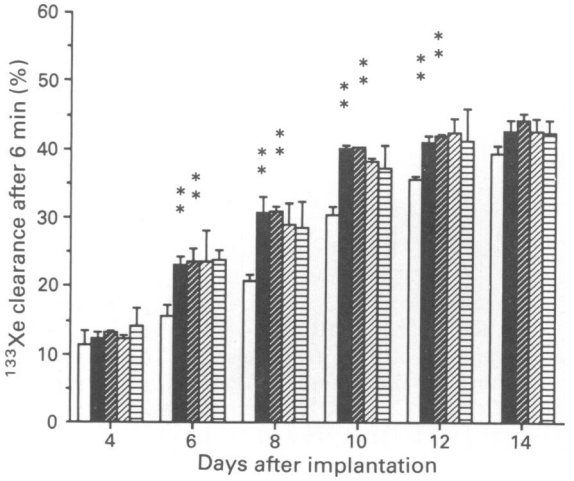

Figure 6 Effect of RP 67580 on angiogenesis induced by interleukin-1α (IL-1α) or bFGF alone. Columns represent sponges treated daily with PBS alone (□), 3 pmol IL-1α (■), 6 pmol bFGF (▨) 3 pmol IL-1α plus 1,000 pmol RP 67580 (▨), and 6 pmol bFGF plus 1,000 pmol RP 67580 (▤); see Methods for details. Each point represents mean data ± s.e.mean from 6 animals. $**P<0.01$ (3 pmol IL-1α vs PBS alone; 6 pmol bFGF vs PBS alone). RP 67580 produced no significant effect on sponges receiving IL-1α or bFGF.

Effects of tachykinin receptor agonists and antagonists

Since the angiogenic response elicited by subthreshold doses of substance P and IL-1α was inhibited by concomitant treatment with 1,000 pmol of the substance P antagonist, [D-Pro^2,D-Phe^7,D-Trp^9]substance P (Figure 3), it was decided to characterize the tachykinin receptors which mediate the angiogenic activity of substance P. Figure 4 shows that daily injection of 1,000 pmol of the selective NK_1 receptor agonist [Sar^9,Met(O$_2$)^{11}]substance P into sponge implants caused a significant increase in ^{133}Xe clearance as compared with controls. Lower doses (10–100 pmol) of [Sar^9,Met(O$_2$)^{11}]substance P did not induce any significant angiogenic response. However, when combined with a subthreshold dose of IL-1α (0.3 pmol), 10 pmol [Sar^9,Met(O$_2$)^{11}]-substance P was sufficient to produce a strong neovascular response similar to that elicited by either 1,000 pmol [Sar^9,Met(O$_2$)^{11}]substance P or 3 pmol IL-1α alone.

The ability of [Sar^9,Met(O$_2$)^{11}]substance P to mimic the angiogenic effect of substance P suggests that NK_1 receptors are likely to be involved. To test this hypothesis, the NK_1 receptor antagonist L-668,169 was used in initial experiments. Since at least 10,000 pmol L-668,169 would be required to inhibit the angiogenic effects of 1,000 pmol [Sar^9,Met(O$_2$)^{11}]-substance P alone, it was decided to test the ability of the antagonist to inhibit the effect of [Sar^9,Met(O$_2$)^{11}]substance P (10 pmol) and IL-1α (0.3 pmol). Figure 4 shows that L-668,169 (1,000 pmol) was able to reduce the elevated ^{133}Xe clearance values of sponges treated with [Sar^9,Met(O$_2$)^{11}]-substance P (10 pmol) and IL-1α (0.3 pmol) to that of controls. Similarly, daily doses of the non-peptide NK_1 receptor antagonist RP 67580 (1,000 pmol) completely antagonized the effect of substance P/IL-1α (Figures 2d and 5). Figure 6 shows that RP 67580 did not block the action of IL-1α. The specificity of NK_1 blockade was further confirmed by the failure of RP 67580 to block the comparable neovascular responses elicited by bFGF (Figure 6). In another series of experiments, a second non-peptide NK_1 receptor antagonist (±)-CP-96,345 (1,000 pmol day^{-1}) was also able to suppress the angiogenic effect of substance P/IL-1α (Figure 7).

In contrast, daily doses of 1,000 pmol of the selective NK_2 receptor agonist [β-Ala^8]NKA(4-10), or the NK_3 receptor agonist, senktide, did not influence the basal neovascular response in the sponges (data not shown). Since [β-Ala^8]NKA(4-10) and senktide display at least 100 times lower affinity than substance P at NK_1 receptors, they were used at 100 times the subthreshold dose of substance P (i.e., 1,000 pmol) in the study of potential interactions between these peptides and IL-1α. However, the combination of these peptides did not modify the basal neovascularization. Likewise, the NK_2 receptor antagonist L-659,874 (1,000 pmol) was ineffective against the angiogenic effect of substance P/IL-1α (data not shown).

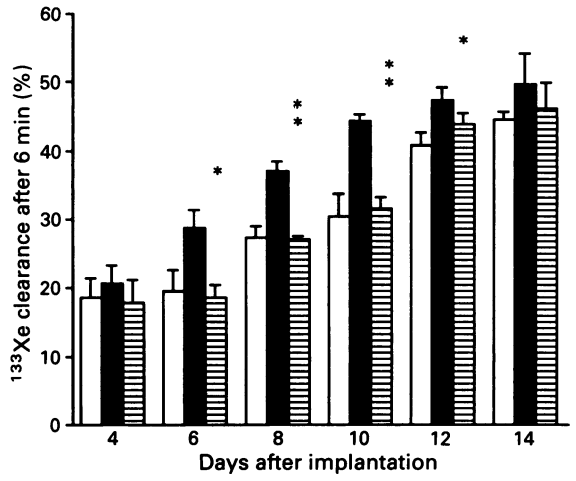

Figure 7 Inhibition of substance P/interleukin-1α (IL-1α)-induced angiogenesis by the non-peptide NK_1 receptor antagonist, (±)-CP-96,345. Columns represent sponges treated daily with PBS alone (□), 10 pmol substance P plus 0.3 pmol IL-1α (■) and 10 pmol substance P plus 0.3 pmol IL-1α plus 1,000 pmol (±)-CP-96,345 (▤); 1,000 pmol (±)-CP-96,345 alone produced no effect; see Methods for details. Each column represents mean data ± s.e.mean from 6–12 animals. $*P<0.05$; $**P<0.01$ (substance P/IL-1α plus (±)-CP-96,345 vs substance P/IL-1α).

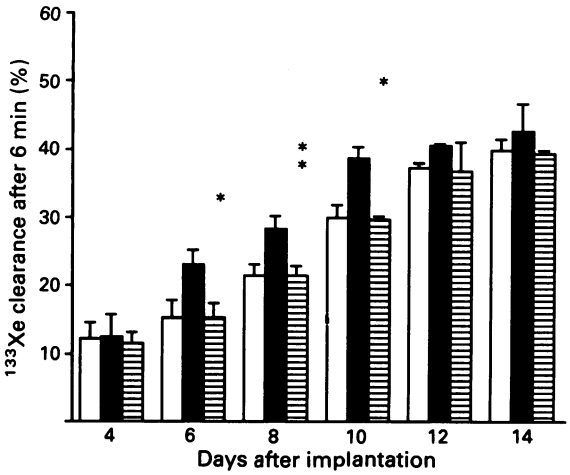

Figure 8 Inhibition of substance P/interleukin-1α (IL-1α)-induced angiogenesis by IL-1 receptor antagonist (IL-1ra). Columns represent sponges treated daily with PBS (□), 10 pmol substance P plus 0.3 pmol IL-1α (■), and 10 pmol substance P plus 0.3 pmol IL-1α plus 50 μg IL-1ra (▤). IL-1ra alone produced no effect; see Methods for details. Each column represents mean data ± s.e.mean from 6 animals. $*P<0.05$; $**P<0.01$ (substance P/IL-1α plus IL-1ra vs substance P/IL-1α).

Effect of IL-1 receptor antagonist

To establish the relative contribution of substance P and IL-1 in the substance P/IL-1α-induced neovascularization, the effect of an IL-1 receptor antagonist (IL-1ra) was examined. Like the NK_1 receptor antagonists (L-668,169, RP 67580 and (±)-CP-96,345), daily administration of 50 μg IL-1ra was able to inhibit the angiogenic effect of substance P/IL-1α (Figures 2e and 8).

Effects of indomethacin, a PAF antagonist and histamine antagonists

To analyse the possible involvement of prostaglandins, PAF and histamine in substance P/IL-1α-induced neovascularization, three different classes of drugs were used. On day 14 after implantation, the 6-keto-PGF$_{1\alpha}$ content in the sponges receiving substance P/IL-1α was elevated by almost 19 fold (376 ± 9 pg per sponge, $n = 6$, $P<0.01$) from that in the sponges receiving PBS alone (20 ± 5 pg per sponge, $n = 6$). When the prostaglandin synthesis inhibitor, indomethacin (7 nmol day^{-1}), was co-administered with the peptides into the sponges, the elevated 6-keto-PGF$_{1\alpha}$ level in these sponges was reduced by 95% (23 ± 8 pg per sponge vs 376 ± 9 pg per sponge, $n = 6$, $P<0.01$). However, indomethacin did not modify new vessel formation as determined by ^{133}Xe clearance (data not shown).

In parallel experiments, daily doses of 22 nmol of the PAF antagonist WEB-2086 had no significant effect (data not shown). Similarly, daily doses of 1 nmol of the H_1-receptor antagonist mepyramine and/or 10 nmol of the H_2-receptor antagonist cimetidine produced no changes on the angiogenesis induced by substance P/IL-1α (data not shown).

Discussion

In this paper, we present two lines of evidence which suggest a role for substance P in angiogenesis. First, a variety of selective tachykinin receptor ligands were able to mimic/antagonize the substance P/IL-1α angiogenic response. Thus, it was found that the angiogenic effect of substance P could be mimicked by the NK_1 selective agonist [Sar^9,$Met(O_2)^{11}$]-substance P, but not by the NK_2 or NK_3 selective agonists (β-Ala^8]-NKA(4-10) and senktide, suggesting that the activation of NK_1 receptors is most likely to mediate this effect of substance P. These results are in agreement with the findings of Ziche *et al.* (1990). Two earlier clinical observations also indicate a role of substance P in angiogenesis. In 1987, Hermanson *et al.* noted an increase in superficial skin wounds of substance P-immunoreactive sensory nerve fibres in connection with blood vessel regeneration. Subsequently, Mantyh and colleagues (1988) reported that in surgical specimens obtained from patients with inflammatory bowel diseases, receptor binding sites for substance P, but not neurokinin A or neurokinin B, were expressed in high concentrations by arterioles, venules and regional lymph nodules.

The other major finding of this paper is that the angiogenic action of substance P/IL-1α can be inhibited by the selective NK_1 receptor antagonists RP 67580 (Garret *et al.*, 1991) and (±)-CP-96,345 (Snider *et al.*, 1991), but not by the NK_2 selective antagonist, L-659,874 (Williams *et al.*, 1988). These observations are of potential clinical relevance in view of a recent report that substance P binding sites, with characteristics of NK_1 receptors, are localized on human synovial endothelial cells (Walsh *et al.*, 1992). This suggests that perivascular nerves containing substance P and substance P binding sites are well placed to play a regulatory role in synovial vasculature. However, the breakdown of this regulatory network may occur in rheumatoid arthritis, as suggested by the work of Levine *et al.* (1984), leading to excessive neovascularization. In such a situation, the blockade of NK_1 receptors in the joint may halt or reverse the progression of the disease. This approach may also have therapeutic implication in the future management of other angiogenic diseases, such as atherosclerosis, diabetic retinopathy and cancer.

Questions remain as to the mechanism by which substance P and IL-1α stimulate blood vessel formation. Both peptides have been shown to stimulate collagenase production by fibroblasts (Lotz *et al.*, 1987; see Payan, 1989), which is vital in the dissolution of the basement membrane of pre-existing blood vessels before angiogenesis can take place. They are also mitogenic for vascular endothelial cells *in vitro* (Ziche *et al.*, 1990; Detmar *et al.*, 1992). In addition, recent studies suggest several levels of interaction between substance P and IL-1 in chronic inflammation. Substance P can stimulate the production of IL-1 and other cytokines by human blood monocytes (Lotz *et al.*, 1988). On the other hand, IL-1 has been shown to increase substance P levels under inflammatory conditions and its gene expression in sympathetic neurones (Arai *et al.*, 1990; Freidin & Kessler, 1991; Hart *et al.*, 1991). Our ability to suppress the substance P/IL-1-induced angiogenesis by the blockade of either NK_1 or IL-1 receptor further illustrates the important interactions between these two peptides in chronic inflammatory diseases. However, IL-1 may play a bi-directional role in angiogenesis. It potentiates the effect of some factors such as substance P, but antagonizes the activity of others, e.g. it inhibits bFGF-induced vessel formation, probably due to its ability to decrease the expression of high-affinity bFGF binding sites on endothelium (Cozzolino *et al.*, 1990).

In an *in vivo* situation, additional factors may also be involved. For example, substance P can release histamine from mast cells (Foreman & Jordan, 1983) and PGE_2 from fibroblasts (Lotz *et al.*, 1987). Furthermore, both IL-1 and substance P are capable of stimulating endothelial cells and neutrophils to produce PAF (Bussolino *et al.*, 1986; Brunelleschi *et al.*, 1990), which could in turn stimulate endothelial cell migration and proliferation (Smither & Fan, 1992) and enhance the sponge-induced angiogenesis (Smither & Fan, 1990). However, the failure of histamine receptor antagonists, indomethacin and the PAF antagonist WEB-2086 to modify the substance P/IL-1α angiogenic response in this model indicates that histamine, prostaglandins and PAF do not play an important role.

In conclusion, the positive interaction between substance P and IL-1α described here underlines the potential importance of these two peptides in the angiogenic cascade leading to a variety of diseases characterized by excessive neovascularization. The successful blockade of the angiogenic response elicited by substance P and IL-1 suggests that in diseases associated with chronic release of these two peptides, nonpeptide NK_1 antagonists or IL-1 receptor antagonists could provide an effective treatment. More importantly, this study demonstrates that angiosuppression can be achieved by blocking the activity of angiogenic factors at the receptor level (Fan & Brem, 1992).

This work was supported by the Wellcome Trust and the British Heart Foundation. We thank Mrs Beverly Wilson and Mr Kwok Kei Chan for their help with histology and Ms Jane Glegg for technical assistance.

References

ANDRADE, S.P., FAN, T.-P.D. & LEWIS, G.P. (1987). Quantitative *in vivo* studies on angiogenesis in a rat sponge model. *Br. J. Exp. Pathol.*, **68**, 755–766.

ARAI, K., LEE, F., MIYAJIMA, A., MIYATAKE, S., ARAI, N. & YOKOTA, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.*, **59**, 783–836.

BRAIN, S.D. & WILLIAMS, T.J. (1989). Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin. *Br. J. Pharmacol.*, **97**, 77–82.

BRUNELLESCHI, S., CENI, E., GIOTTI, A. & FANTOZZI, R. (1990). Tachykinins stimulate lyso-PAF:acetyl-CoA acetyltransferase activity in neutrophils. *Eur. J. Pharmacol.*, **186**, 367–368.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991). Inflammatory edema induced by interactions between IL-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146**, 3424–3430.

BUSSOLINO, F., BREVIARIO, F., TETTA, C., AGLIETTA, M., MANTOVANI, A. & DEJENA, E. (1986). Interleukin-1 stimulates platelet-activating factor production in cultured human endothelial cells. *J. Clin. Invest.*, **77**, 2027–2033.

CASALS-STENZEL, J., MUACEVIC, G. & WEBER, K.-H. (1987). Pharmacological actions of WEB 2086, a new specific antagonist of platelet-activating factor. *J. Pharmacol. Exp. Ther.*, **241**, 974–981.

COZZOLINO, F., TORCIA, M., ALDINUCCI, D., ZICHE, M., ALMERIGOGNA, F., BANI, D. & STERN, D.M. (1990). Interleukin 1 is an autocrine regulator of human endothelial cell growth. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 6487–6491.

DETMAR, M., TENORIO, S., HETTMANNSPERGER, U., RUSZCZAK, Z. & ORFANOS, C.E. (1992). Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells in vitro. *Invest. Dermatol.*, **98**, 147–153.

DRAPEAU, G., D'ORLEANS-JUSTE, P., DION, S., RHALEB, N.-E. & REGOLI, D. (1987). Selective agonists for substance P and neurokinin receptors. *Neuropeptides*, **10**, 43–54.

FAN, T.-P.D. & BREM, S. (1992). Angiosuppression. In *The Search for New Anticancer Drugs*. ed. Waring, M.J. & Ponder, B.A.J. pp. 183–227. Dordrecht, Boston, London: Kluwer Academic Publishers.

FAN, T.-P.D. & HU, D.-E. (1991). Modulation of angiogenesis by inflammatory polypeptides. *Int. J. Radiat. Biol.*, **60**, 71.

FAN, T.-P.D., HU, D.-E. & HILEY, C.R. (1992a). Development and validation of a sponge model for quantitative studies on angiogenesis. In *Angiogenesis in Health and Disease*, pp. 317–332. ed. Maragoudakis, M.E., Gullino, P. & Lelkes, P.I. New York & London: Plenum Press.

FAN, T.-P.D., HU, D.-E., SMITHER, R.L. & GRESHAM, G.A. (1992b). Further studies on angiogenesis in a rat sponge model. In: *Angiogenesis*, pp. 308–314. ed. Steiner, R., Weisz, P.B. & Langer, R. Basel: Birkhaüser Verlag AG.

FAN, T.-P.D. & LEWIS, G.P. (1984). Effect of cyclosporin A and inhibitors of arachidonic acid metabolism on blood flow and cyclo-oxygenase products in rat skin allografts. *Br. J. Pharmacol.*, **81,** 261–271.

FOLKMAN, J. & KLAGSBRUN, M. (1987). Angiogenic factors. *Science*, **235,** 442–447.

FOREMAN, J.C. & JORDAN, C.C. (1983). Histamine release and vascular changes induced by neuropeptides. *Agents Actions*, **13,** 105–116.

FREIDIN, M. & KESSLER, J.A. (1991). Cytokine regulation of substance P expression in sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 3200–3203.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAQUI, S., PEYRONEL, J.-F., BLANCHARD, J.-C. & LADURON, P.M. (1991). Pharmacological properties of a potent selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10208–10212.

GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor types: classification and membrane signalling mechanisms. *Neurochem. Int.*, **18,** 149–165.

HAEGERSTRAND, A., DALSGAARD, C.-J., JONZON, B., LARSSON, O. & NILSSON, J. (1990). Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 3299–3303.

HANNUM, C.H., WILCOX, C.J., AREND, W.P., JOSLIN, F.G., DRIPPS, D.J., HEIMDAL, P.L., ARMES, L.G., SOMMER, A., EISENBERG, S.P. & THOMPSON, R.C. (1990). Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature*, **343,** 336–340.

HART, R.P., SHADIACK, A.M. & JONAKAIT, G.M. (1991). Substance P gene expression is regulated by interleukin-1 in cultured sympathetic ganglia. *J. Neurosci. Res.*, **29,** 282–291.

HERMANSON, A., DALSGAARD, C.-J., BJÖRKLUND, H. & LINDBLOM, U. (1987). Sensory reinnervation and sensitivity after superficial skin wounds in human patients. *Neurosci. Lett.*, **74,** 377–382.

HU, D.-E. & FAN, T.-P.D. (1991). Synergistic interaction between bradykinin and interleukin-1 in angiogenesis. *Br. J. Pharmacol.*, **104,** 83P.

HU, D.E. & FAN, T.-P.D. (1993). [Leu^8]des-Arg^9-bradykinin inhibits the angiogenic effect of bradykinin and interleukin-1 in rats. *Br. J. Pharmacol.*, **109,** 14–17.

HU, D.E., HILEY, C.R. & FAN, T.-P.D. (1992). Parallel studies of ^{133}Xe clearance and ^{113}Sn microsphere accumulation during sponge-induced angiogenesis and the effects of interleukin-1α. *Br. J. Pharmacol.*, **106,** 102P.

KIMBALL, E.S. & FISHER, M.C. (1988). Potentiation of IL-1-induced BALB/3T3 fibroblast proliferation by substance P. *Ann. N.Y. Acad. Sci.*, **540,** 681–683.

KLAGSBRUN, M. & D'AMORE, P.A. (1991). Regulators of angiogenesis. *Annu. Rev. Physiol.*, **53,** 217–239.

LEVINE, J.D., CLARK, R., DEVOR, M., HELMS, C., MOSKOWITZ, M.A. & BASBAUM, A.I. (1984). Intraneuronal substance P contributes to the severity of experimental arthritis. *Science*, **226,** 547–549.

LOTZ, M., CARSON, D.A. & VAUGHAN, J.H. (1987). Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. *Science*, **235,** 893–895.

LOTZ, M., VAUGHAN, J.H. & CARSON, D.A. (1988). Effects of neuropeptides on production of inflammatory cytokines by human monocytes. *Science*, **241,** 1218–1221.

MANTYH, C.R., GATES, T.S., ZIMMERMAN, R.P., WELTON, M.L., PASSARO, E.P. Jr., VIGNA, S.R., MAGGIO, J.E., KRUGER, L. & MANTYH, P.W. (1988). Receptor binding sites for substance P, but not substance K or neuromedin K, are expressed in high concentrations by arterioles, venules, and lymph nodules in surgical specimens obtained from patients with ulcerative colitis and Crohn disease. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 3235–3239.

MOSES, M.A. & LANGER, R. (1991). Inhibitors of angiogenesis. *Biotechnology*, **9,** 630–634.

NILSSON, J., VON EULER, A.M. & DALSGAARD, C.-J. (1985). Stimulation of connective tissue cell growth by substance P and substance K. *Nature*, **315,** 61–63.

PAYAN, D.G. (1989). Neuropeptides and inflammation: the role of substance P. *Ann. Rev. Med.*, **40,** 341–352.

ROVERO, P., PESTELLINI, V., RHALEB, N.-E., DION, S., ROUISSI, N., TOUSIGNANT, C., TELEMAQUE, S., DRAPEAU, G. & REGOLI, D. (1989). Structure-activity studies of neurokinin A. *Neuropeptides*, **13,** 263–270.

SMITHER, R.L. & FAN, T.-P.D. (1990). PAF antagonists inhibit angiogenesis in a rat sponge model. *Br. J. Pharmacol.*, **99,** 87P.

SMITHER, R.L. & FAN, T.-P.D. (1992). Effects of platelet-activating factor on endothelial cells and fibroblast in vitro. In: *Angiogenesis*, pp. 230–234. ed. Steiner, R., Weisz, P.B. & Langer, R. Basel: Birkhaüser Verlag AG.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, J.A., III, LONGO, K.P. LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991). A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science*, **251,** 435–437.

WALSH, D.A., MAPP, P.I., WHARTON, J., RUTHERFORD, R.A.D., KIDD, B.L., REVELL, P.A., BLAKE, D.R. & POLAK, J.M. (1992). Localisation and characterisation of substance P binding to human synovial tissue in rheumatoid arthritis. *Ann. Rheum. Dis.*, **51,** 313–317.

WILLIAMS, B.J., CURTIS, N.R., MCKNIGHT, A.T., MAGUIRE, J., FOSTER, A. & TRIDGETT, R. (1988). Development of NK-2 selective antagonists. *Regul. Pept.*, **22,** 189.

WORMSER, U., LAUFER, R., HART, Y., CHOREV, M., GILON, C. & SELINGER, Z. (1986). Highly selective agonists for substance P receptor subtypes. *EMBO J.*, **5,** 2805–2808.

ZICHE, M., MORBIDELLI, L., PACINI, M., GEPPETTI, P., ALESSANDRI, G. & MAGGI, C.A. (1990). Substance P stimulates neovascularization *in vivo* and proliferation of cultured endothelial cells. *Microvasc. Res.*, **40,** 264–278.

(Received February 25, 1993
Accepted May 10, 1993)