Immunology 1989 **68** 359–364

Activation of human neutrophils by substance P: effect on FMLP-stimulated oxidative and arachidonic acid metabolism and on antibody-dependent cell-mediated cytotoxicity

A. WOZNIAK, G. MCLENNAN, W. H. BETTS,* G. A. MURPHY* & R. SCICCHITANO
*Department of Thoracic Medicine, Royal Adelaide Hospital, Adelaide and *Rheumatology Unit, the Queen Elizabeth Hospital, Woodville, South Australia*

Accepted for publication 3 July 1989

SUMMARY

We show that the neuropeptide, substance P (SP), a putative mediator of neurogenic inflammation, is a potent regulator of mature, human neutrophil function. SP increased neutrophil cytotoxic activity against an antibody-coated target (P815 cells) in a dose-dependent manner. The maximal effect was noted at an SP concentration of 10^{-4} M, when cytotoxicity increased from $4{\cdot}7 \pm 0{\cdot}9\%$ to $33{\cdot}4 \pm 10{\cdot}3\%$. This effect was not due to toxicity of SP against the target cells and was antibody-dependent. The level of cytotoxic activity induced by SP was comparable to that described for a number of cytokines, such as GM-CSF, under identical assay conditions. SP-induced cytotoxicity was $73{\cdot}1 \pm 5{\cdot}8\%$ of that produced by an optimum concentration of conditioned medium known to contain a number of cytokines which activate mature neutrophils. In addition, SP enhanced FMLP-stimulated superoxide anion production by neutrophils in a dose-dependent fashion. Neutrophils preincubated with medium or $7{\cdot}5 \times 10^{-5}$ M SP and then stimulated with 10^{-7} M FMLP produced $7{\cdot}9 \pm 2{\cdot}7$ and $29{\cdot}9 \pm 3{\cdot}7$ nmol superoxide anion/10^6 cells, respectively. This priming effect of SP was rapid in onset (< 15 min) and was maximal from 15 to 60 min, after which it declined. It was not reversed by washing the cells and was temperature dependent. SP did not shift the dose–response curve to FMLP to the left, but it enhanced the response to FMLP in the concentration range 10^{-8}–10^{-6} M. Similarly SP enhanced LTB_4 and 5-HETE production by FMLP-stimulated but not calcium ionophore-stimulated neutrophils. Therefore, these data provide evidence that SP regulates a number of neutrophil functions and suggests a mechanism whereby the nervous system may affect the immune response. Furthermore, the regulatory effects of SP on the neutrophil functions studied appear to be similar to those of a number of cytokines that have been previously implicated in inflammation.

INTRODUCTION

The undecapeptide substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was originally isolated from the equine brain, but is now known to be distributed widely in the central and peripheral nervous systems of many species, including man (Pernow, 1983; Holzer, 1988). In the peripheral nervous system it is synthesized in dorsal root ganglion cells and it is present mainly in C-afferent fibres, where it is thought to act as a neurotransmitter.

A number of studies have suggested that SP is involved in the inflammatory response, so called neurogenic inflammation, following its release by local axon reflexes (Jancso, Jancso-Gabor & Szolcsanyi, 1967; Thureson-Klein *et al.*, 1987). SP has a number of actions on non-neuronal tissues which lend support to this role as a chemical mediator of inflammation and as a link between the nervous system and the immune response: it induces a vasodilator response and causes tissue oedema (Lundberg *et al.*, 1983) and it has direct effects on a number of inflammatory cells (reviewed by Payan, McGillis & Goetzl, 1986).

SP also has a regulatory role by stimulating the release of cytokines such as IL-1 and IL-6 (Lotz, Vaughan & Carson, 1988) and interferon-gamma (IFN-γ) (Wagner *et al.*, 1987), as well as inflammatory mediators including histamine (Shanahan *et al.*, 1985) and prostaglandins (Hartung, Wolters & Toyka, 1986), all of which are important in the acute-phase reaction.

Abbreviations: AA, arachidonic acid; ADCC, antibody-dependent cell-mediated cytotoxicity; BCM, bladder-conditioned medium; DPBS, modified Dulbecco's phosphate-buffered saline; FMLP, formyl-methionyl-leucyl-phenylalanine; GM-GSF, granulocyte–macrophage colony-stimulating factor; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTB_4, leukotriene B_4; O_2^-, superoxide anion; SOD, superoxide dismutase; SP, substance P; TNF, tumour necrosis factor; TNP, trinitrophenyl.

Correspondence: Dr R. Scicchitano, Dept. of Thoracic Medicine, Royal Adelaide Hospital, North Terrace, Adelaide, South Australia 5000.

In the skin, SP has been implicated in the wheal and flare reaction in response to a number of noxious stimuli as well as antigen challenge (Foreman & Jordan, 1983). All of these findings have led to the hypothesis that SP is the chemical mediator of inflammation in a number of diseases, including asthma (Barnes, 1986) and rheumatoid arthritis (Lotz, Carson & Vaughan, 1987; Levine *et al.*, 1984).

In order to define the role of SP in inflammation more precisely, we investigated its effects on human neutrophil function. We report that SP stimulates neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC) but that it has little direct effect on superoxide anion (O_2^-) production or metabolism of arachidonic acid (AA) via the 5-lipo-oxygenase pathway. However, SP primed neutrophils to produce increased amounts of O_2^-, leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) in response to the stimulus formyl-methionyl-leucyl-phenylalanine (FMLP). This is the first report, to our knowledge, that SP acts as a priming agent for neutrophils.

MATERIALS AND METHODS

Isolation of human neutrophils

Neutrophils for ADCC and O_2^- production were isolated from the peripheral blood of normal volunteers who did not suffer from clinical asthma or allergic rhinitis. A leucocyte fraction was obtained by sedimenting the erythrocytes with dextran (Dextran T-500; Pharmacia, Uppsala, Sweden). Neutrophils were isolated by density-gradient centrifugation (400 ***g***, 20 min, 22°) using Lymphoprep (NYCOMED AS, Oslo, Norway) and hypotonic lysis of residual erythrocytes. The cells were always >96% pure, as determined by Grunwald–Giemsa staining, and >98% viable by trypan blue exclusion. Neutrophils were resuspended either in RPMI-1640 medium containing 20 mM HEPES (Gibco, Grand Island, NY), 2 mM L-glutamine, 60 μg/ml penicillin and 8 μg/ml gentamycin for the ADCC assay, or in a modified Dulbecco's phosphate-buffered saline (DPBS; Gibco; 0·5 mM Mg^{2+}, 0·5 mM Ca^{2+}, 0·75 mM glucose, pH 7·3) for measurement of O_2^- production. For assessment of LTB_4 and 5-HETE production, neutrophils were isolated using Percoll as described previously (McColl *et al.*, 1986). Briefly, erythrocytes were sedimented with dextran and the neutrophils were purified on a double Percoll gradient, specific gravities 1·070 and 1·092 (Pharmacia), at 450 ***g*** for 20 min at 22°. Cells were collected from the interface between the two Percoll layers, washed in DPBS (Ca^{2+} and Mg^{2+} free) and resuspended in DPBS.

Antibody-dependent cell-mediated cytotoxicity

The method had been described in detail previously (Vadas, Nicola & Metcalf, 1983). All experiments were performed in triplicate and SD were < 10% in each experiment. The assay was performed in RPMI-1640 containing 0·1% bovine serum albumin (BSA). Briefly, 40 μl (containing 4×10^3) of ^{51}Cr-labelled trinitrophenyl (TNP)-coupled P815 target cells (DBA/2 mastocytoma; a gift from Dr Angel Lopez, Institute Medical and Veterinary Science, Adelaide) were mixed with 80 μl ($1{\cdot}2 \times 10^5$) neutrophils as effector cells (target:effector ratio (1:30) and 24 μl of rabbit IgG anti-TNP (Miles-Yeda, Rehovot, Israel) and 16 μl of SP (final concentrations 10^{-7}, 10^{-6}, 10^{-5}, $2{\cdot}5 \times 10^{-5}$, 5×10^{-5}, $7{\cdot}5 \times 10^{-5}$ and 10^{-4} M) (AUSPEP, Melbourne) in V-bottomed microtitre plates. The final volume was 160 μl. After incubation for 2·5 hr at 37°, 80 μl of the supernatant were removed and the radioactivity counted using a gamma-counter (LKB, Turku, Finland, 1282 Commugamma, Universal gamma counter).

Percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental c.p.m.} - \text{spontaneous release c.p.m.}}{\text{total c.p.m.} - \text{spontaneous release c.p.m.}} \times 100$$

where spontaneous release was the ^{51}Cr released from P815 cells in the presence of medium alone and the total count was the ^{51}Cr released from P815 cells lysed by the addition of 4% Triton X-100.

We used bladder-conditioned medium (BCM) from the bladder carcinoma cell line, U5637 (a gift from Dr Angel Lopez) as a positive control since it contains a number of colony-stimulating factors (CSF) known to enhance human neutrophil ADCC (Vadas *et al.*, 1983; Lopez *et al.*, 1986).

Superoxide production

Generation of O_2^- was measured as superoxide dismutase (SOD)-inhibitible reduction of ferricytochrome C. This was determined by addition of SOD (10 μl of 2 mg/ml; Boehringer Mannheim, GMbH, FRG) to duplicate samples in all experiments. In the initial dose–response experiments, 10^6 neutrophils were incubated in triplicate with cytochrome C (Calbiochem-Behring, N.S.W.; final concentration 10^{-4} M) with medium or varying concentrations of SP (10^{-9}–10^{-4} M) in a final volume of 1·0 ml. Cells were incubated for 30 min at 37° and then FMLP or medium was added (10 μl; final concentration 10^{-7} M) and the mixture incubated for a further 6 min. The reaction was stopped by addition of SOD (10 μl of 2 mg/ml) and by immersion of tubes in ice water (4°). The cells were pelleted by centrifugation at 4°. O_2^- production was measured by reduction of cytochrome C by measuring absorption at 550 nm (Weening, Weever & Roos, 1975) and using an extinction coefficient of 21·1 nM^{-1} cm^{-1} (Van Gelder & Slater, 1962). In subsequent experiments (kinetics, temperature-dependence and effect on FMLP dose–response curve), purified neutrophils (5×10^6/ml) were incubated in triplicate with medium or different concentrations of SP for the stated times at 37°. Then, 200 μl of cells (10^6) were removed and added to 800 μl of a mixture containing cytochrome C (10^{-4} M) and FMLP (at stated concentrations). This reaction mixture was then incubated for 6 min at 37° and the reaction was stopped as outlined above.

LTB_4 and 5-HETE production

Neutrophils isolated by Percoll density-gradient centrifugation were washed and resuspended in DPBS (10^6 cells/ml). Nine-hundred microlitres of cells were incubated with medium or SP (10^{-7} and 5×10^{-5} M) for 15 min at 37°, and were then stimulated with 0·5 μM A23187 (Sigma, St Louis, MO) or 10^{-7} M FMLP (Sigma) for 5 min at 37° in the presence or absence of 10^{-5} M arachidonic acid (AA; Sigma). The reaction was stopped by addition of 250 μl of 100 mM citric acid, which reduced the pH to 3. Internal standards [Prostaglandin B_2 (Sigma) and 15-HETE, synthesized according to the method of McColl *et al.* (1986)] were added to the samples followed by extraction with a chloroform–methanol mixture (7:3). The organic phase was collected, evaporated to dryness under vacuum using a centrifugal evaporator (Savant, Hicksville, NY) and the samples were

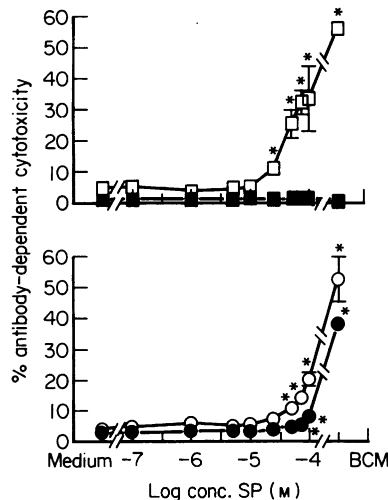

Figure 1. The effect of SP on neutrophil cytotoxicity. Neutrophils were incubated with various concentrations of SP (10^{-7}–10^{-4} M) or medium as indicated for 2·5 hr with the indicated dilutions of anti-TNP antibody (■, 0 antibody; □, 1/300; ○, 1/1000; ●, 1/3000). Data are means ± SEM of four experiments performed in triplicate. * Indicates values (in presence of SP) which differ significantly from the corresponding medium control ($P<0·05$–$P<0·01$).

reconstituted in 100 µl of methanol. Aliquots of 25 µl were analysed with each run. LTB_4 and 5-HETE were measured by reversed-phase high-performance liquid chromatography (HPLC) as described previously (McColl *et al.*, 1986).

HPLC analysis

The HPLC system was from Waters Millipore (Milford, MA) and included a model 510 pump, 730 data module, 490 variable wavelength UV detector and 710B WISP autoinjector. The mobile phase was methanol–water–acetic acid (70:30:0·08) and the pH was 6·2 (adjusted with ammonium hydroxide). The separation was done using a C_{18} Nova Pak column (Waters Millipore) and guard column at a flow-rate of 1·5 ml/min. Detection of LTB_4 was carried out at 270 nm and 5-HETE at 235 nm.

Statistics

Data were analysed using Student's paired *t*-test and analysis of variance or non-parametric statistics (Mann–Whitney *U* test) as indicated, using a MacIntosh Plus computer and the Statview 512™ (1986) statistics package. Results are expressed as mean ± SEM (*n* = number of experiments).

RESULTS

Effect of SP on ADCC

SP stimulated neutrophil cytotoxicity in a dose-dependent manner at all antibody concentrations tested (Fig. 1). Optimal antibody dilution was 1/300. At higher antibody concentrations, high levels of cytotoxicity were seen in the absence of a stimulus in some individuals. At this antibody dilution, a significant effect was noted at an SP concentration of $2·5 \times 10^{-5}$ M ($11·1 \pm 1·6$% versus $4·7 \pm 0·9$%, $n=4$, $P<0·02$, compared to medium control). Maximal mean cytotoxicity was observed at 10^{-4} M SP ($33·4 \pm 10·3$%, $n=4$, $P<0·05$). However, in one

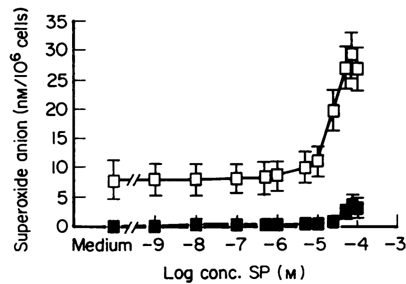

Figure 2. The effect of SP on superoxide production. Neutrophils (10^6/ml) were incubated with cytochrome C and the stated concentrations of SP (10^{-9}–10^{-4} M) or medium control for 30 min at 37°. They were then stimulated with 10^{-7} M FMLP (□) or diluent (■) for 6 min. Data are means ± SEM of four experiments each performed in triplicate.

subject maxmum cytotoxicity (37·5%) was seen at an SP concentration of 5×10^{-5} M, while it occurred at 10^{-4} M in the remaining three (34, 39 and 55%). Since the magnitude of the response to SP differed between individuals, data were also calculated as a percentage of ADCC stimulated by an optimum dilution of BCM (1/10). In these experiments mean maximal cytotoxicity induced by SP was $73·1 \pm 5·8$% of that induced by BCM (range 64·2–88·7%).

At antibody dilutions of 1/3000 and 1/1000 a small but significant effect was noted at an SP concentration of 5×10^{-5} M compared to medium control ($4·5 \pm 0·6$% and $10·9 \pm 1·9$% versus $2·4 \pm 1·0$% and $3·4 \pm 0·2$%, $P<0·02$ and $P<0·04$, respectively). Maximum effects were seen at an SP concentration of 10^{-4} M ($8·1 \pm 1·4$% and $20·6 \pm 2·2$%, $P<0·05$ and $P=0·005$, respectively).

Spontaneous release of ^{51}Cr from target cells was < 10%. No significant cytotoxicity was noted in the absence of antibody or neutrophils. In the absence of SP or BCM, cytotoxicity was < 7% at all antibody concentrations tested. SP alone exhibited no cytotoxicity on the target cells.

Effect of SP on superoxide production

In initial experiments, 10^6 neutrophils were incubated with cytochrome C and SP (10^{-9}–10^{-4} M) or control medium for 30 min at 37°. At the end of this period cells were stimulated by the addition of 10^{-7} M FMLP or control diluent and O_2^- production measured after 6 min. Data are shown in Fig. 2. SP alone (without subsequent stimulation with FMLP) induced a small but significant production of O_2^- ($3·6 \pm 1·6$ nmol/10^6 cells at $7·5 \times 10^{-5}$ M SP, $n=4$). This represented 48 ± 15% of the response induced by 10^{-7} M FMLP. Preincubation with SP greatly enhanced FMLP-stimulated O_2^- production in a dose-dependent fashion (Fig. 2). A small priming effect was noted at 10^{-5} M ($11·0 \pm 3·6$ nmol O_2^-/10^6 cells, $P<0·03$) and the maximum effect was noted at $7·5 \times 10^{-5}$ M SP ($29·1 \pm 3·7$ versus $7·9 \pm 2·7$ nmol O_2^-/10^6 cells for SP and medium control, respectively, $P<0·01$). At this optimum concentration SP enhanced the response to FMLP approximately five-fold (480 ± 146%, range 252–906%).

In order to study the kinetics of this priming effect, neutrophils (5×10^6/ml) were incubated with 5×10^{-5} M SP at 37° for varying times (1–120 min). At each time point, 10^6 neutrophils were placed into tubes containing 10^{-7} M FMLP (final concentration) and cytochrome C. The reaction was

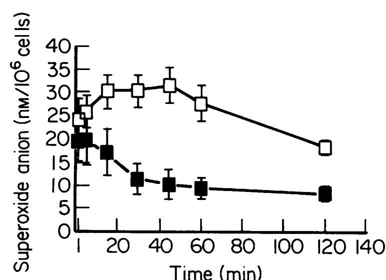

Figure 3. The effect of SP on FMLP-stimulated superoxide production as a function of time. Neutrophils (5×10^6/ml) were incubated with medium (■) or 5×10^{-5} M SP (□) at 37° for the indicated times. At each time point 10^6 neutrophils were added to tubes containing cytochrome C and 10^{-7} M FMLP and O_2^- production measured. Values are mean ± SEM from four experiments each performed in triplicate.

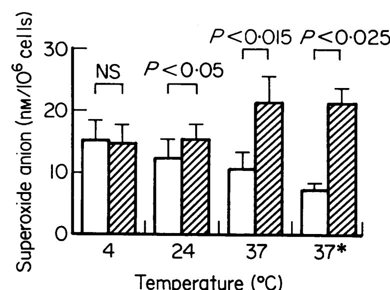

Figure 5. Temperature dependence of SP on FMLP-stimulated superoxide production. Neutrophils were incubated with SP 5×10^{-5} M (■) or medium (□) for 30 min at the stated temperature, washed, and then stimulated with 10^{-7} M FMLP at 37°. Data are means ± SEM from four experiments each performed in triplicate. 37* refers to cells which were incubated with SP or medium at 37° and then stimulated with FMLP without prior washing. Values which differed significantly are shown.

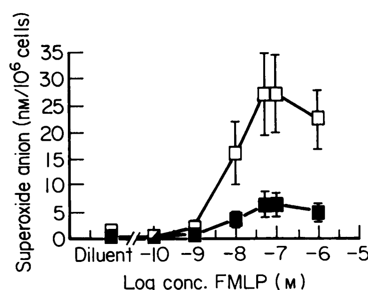

Figure 4. Effect of SP on the superoxide dose-response to FMLP. Neutrophils were incubated with 5×10^{-5} M SP (□) or medium (■) for 30 min and were then stimulated with the stated concentrations of FMLP or diluent for 6 min. Values are mean ± SEM from four experiments each performed in triplicate.

stopped at 6 min. Results are depicted in Fig. 3. The priming effect of SP on FMLP-stimulated O_2^- production was already evident at 5 min, plateaued between 15 and 60 min ($P < 0{\cdot}05$–$< 0{\cdot}0025$) then declined slowly, but was still significant at 120 min ($P < 0{\cdot}02$, Fig. 3). In these experiments there was no significant O_2^- production when neutrophils were stimulated with SP alone for 6 min.

The effect of SP on the O_2^- dose-response curve to FMLP was also investigated (Fig. 4). SP enhanced the response to FMLP at all doses tested between 10^{-8} and 10^{-6} M FMLP ($P < 0{\cdot}05$–$0{\cdot}02$), but did not shift the FMLP dose-response curve to the left.

To test the temperature dependence of the priming effect of SP, neutrophils were incubated with 5×10^{-5} M SP or medium for 30 min at 4° (on ice), 24° or 37°. They were then washed twice in DPBS, allowed to equilibrate to 37°, and then stimulated with 10^{-7} M FMLP for 6 min. Data are shown in Fig. 5. The priming effect was not evident when cells were incubated with SP at 4°. Cells incubated at 24° and 37° showed an enhanced response to FMLP from $12{\cdot}3 \pm 3{\cdot}1$ to $15{\cdot}4 \pm 2{\cdot}3$ nmol $O_2^-/10^6$ cells ($n = 4$, $P < 0{\cdot}05$) and $10{\cdot}7 \pm 2{\cdot}6$ to $21{\cdot}3 \pm 4{\cdot}3$ nmol $O_2^-/10^6$ cells ($P < 0{\cdot}015$), respectively. Furthermore the effect of SP was not reversed by washing the cells after incubation. When cells were incubated with SP at 37° the response after washing before stimulation with FMLP did not differ to that when cells were stimulated without washing (Fig. 5).

Table 1. The effect of SP on LTB_4 and 5-HETE production by neutrophils stimulated with FMLP or A23187 with or without AA

Conc. of SP (M)	Stimulus	AA	5-HETE (ng/10^6 cells)	LTB_4 (ng/10^6 cells)
0 (medium)	A23187	−	$29{\cdot}8 \pm 4{\cdot}2$	$7{\cdot}3 \pm 1{\cdot}0$
0 (medium)		+	$104{\cdot}7 \pm 13{\cdot}2$	$12{\cdot}3 \pm 1{\cdot}4$
10^{-7}	A23187	−	$30{\cdot}6 \pm 8{\cdot}7$	$6{\cdot}0 \pm 1{\cdot}2$
10^{-7}		+	$110{\cdot}1 \pm 11{\cdot}6$	$12{\cdot}1 \pm 2{\cdot}0$
5×10^{-5}	A23187	−	$43{\cdot}2 \pm 12{\cdot}1$	$8{\cdot}7 \pm 2{\cdot}0$
5×10^{-5}		+	$117{\cdot}8 \pm 10{\cdot}7$	$14{\cdot}5 \pm 2{\cdot}7$
0 (medium)	FMLP	−	ND	ND
0 (medium)		+	$23{\cdot}4 \pm 6{\cdot}2$*	$3{\cdot}5 \pm 0{\cdot}8$†
10^{-7}	FMLP	−	ND	ND
10^{-7}		+	$26{\cdot}7 \pm 6{\cdot}8$	$4{\cdot}3 \pm 1{\cdot}1$
5×10^{-5}	FMLP	−	ND	ND
5×10^{-5}			$43{\cdot}1 \pm 7{\cdot}4$*	$7{\cdot}0 \pm 1{\cdot}4$†

Neutrophils (10^6/ml) were incubated with medium or SP for 15 min and then stimulated by addition of 5 μM A23187 or 10^{-7} M FMLP with or without AA (10 μM). Data are means ± SEM (three experiments performed in triplicate).

Values with the same superscript (*, †) are significantly different from each other ($P < 0{\cdot}05$).

ND, not detected.

± Refers to presence or absence of AA.

Effect on LTB_4 and 5-HETE production

The effect of SP on the production of the metabolites of the 5-lipo-oxygenase pathway, LTB_4 and 5-HETE, was studied by incubating cells with medium or SP (10^{-7} or 5×10^{-5} M) for 15 min followed by stimulation by 0·5 μM A23187 or 10^{-7} M FMLP for 5 min in the presence or absence of 10 μM exogenous AA. As can be seen from Table 1, SP did not significantly affect the production of LTB_4 and 5-HETE by neutrophils stimulated with A23187, but did increase the response to FMLP plus AA ($P < 0{\cdot}05$). LTB_4 production increased from $3{\cdot}5 \pm 0{\cdot}8$ to $7{\cdot}0 \pm 2{\cdot}4$

ng/10^6 neutrophils ($n = 3$) after preincubation with 5×10^{-5} M SP and stimulation with FMLP plus AA; 5-HETE production increased from $23 \pm 10{\cdot}7$ to $43 \pm 12{\cdot}8$ ng/10^6 cells. SP alone did not stimulate any LTB_4 or 5-HETE production (data not shown).

DISCUSSION

The present study shows that SP regulates human neutrophil function in a complex and selective manner: It enhances neutrophil effector activity by directly stimulating ADCC and to a lesser extent O_2^- production. A much more dramatic effect is to enhance the production of inflammatory mediators (LTB_4, 5-HETE and O_2^-) following stimulation with FMLP. These are neutrophil functions which are thought to have an important role in the pathogenesis of inflammation. Therefore, on the basis of these studies, and the work of others, SP appears to be a potent pro-inflammatory agent able to stimulate the production of oxy radicals and AA metabolites, to degranulate neutrophils (Marasco, Showell & Becker, 1981) and to stimulate neutrophil chemotaxis and phagocytosis (Bar-Shavit *et al.*, 1980; Marasco *et al.*, 1981). We show that it is also able to prime neutrophils for an enhanced response to a second stimulus. Furthermore, these effects of SP on neutrophil function are qualitatively and quantitatively similar to those of a number of cytokines such as GM-CSF and tumour necrosis factor (TNF) under the same assay conditions (Vadas *et al.*, 1983; Lopez *et al.*, 1986; Atkinson *et al.*, 1988 a,b). For example, Lopez *et al.* (1986) reported that GM-CSF increased neutrophil ADCC from 6% to 32%, comparable to the levels induced by SP.

SP stimulated ADCC by human neutrophils. This effect was not due to the toxic effect of SP on the target cells and was dependent on the antibody concentration. Spontaneous cytotoxicity (in the absence of antibody) was not enhanced. The mechanisms by which neutrophils lyse antibody-coated target cells are uncertain: contact between the effector and target cells is necessary and neutrophil degranulation may also be important (Dallegri *et al.*, 1987). A possible mechanism by which SP may stimulate ADCC is to increase the expression of neutrophil Fc gamma receptors or to degranulate neutrophils. Production of oxy radicals may also be important: although SP alone induced little O_2^- production by neutrophils, it is possible that SP also primes the O_2^- response to Fc stimulation. We are currently performing experiments to determine the mechanisms involved. Several other studies have shown that SP affects neutrophil opsonin-dependent phagocytosis and degranulation (Bar-Shavit *et al.*, 1980).

SP enhanced the production of mediators including O_2^-, LTB_4 and 5-HETE following stimulation with FMLP. Serra *et al.* (1988) reported that SP stimulated neutrophils to produce H_2O_2, an effect noted at SP concentrations greater than 10^{-4} M. We showed a small direct effect of SP on O_2^- production, but no direct effect on AA metabolism via the 5-lipo-oxygenase pathway.

The mechanism(s) by which SP primes the neutrophil response to FMLP is (are) uncertain. Marasco *et al.* (1981) reported that in rabbits, SP stimulated neutrophil chemotaxis and caused degranulation, most likely by binding to the FMLP receptors. In our experiments this is an unlikely mechanism since SP potentiated O_2^- production in response to FMLP. If SP were binding to the human FMLP receptor, this would be expected to interfere with the subsequent response to FMLP. Furthermore recent evidence suggests that SP acts via a biochemical pathway which differs from that induced by FMLP (Serra *et al.*, 1988).

The priming effect of SP on O_2^- production was rapid in onset (< 15 min) and the FMLP dose–response curve was not shifted to the left. A possible mechanism by which SP exerts its effects may be to mobilize intracellular calcium with subsequent translocation of protein kinase C to the cell membrane. SP increases phosphoinositide turnover in brain (Watson & Downes, 1983) and human neutrophils (Serra *et al.*, 1988) and mobilizes intracellular calcium in neutrophils (Serra *et al.*, 1988) and smooth muscle cells (Payan, 1985). This could have the effect of increasing the intrinsic responsiveness to FMLP, an agonist which appears to act through the phospholipase C pathway (Allen *et al.*, 1988; Omann *et al.*, 1987). A more likely mechanism is that SP modulates the neutrophil FMLP receptor in much the same way as reported for TNF (Atkinson *et al.*, 1988b) and GM-CSF (Atkinson *et al.*, 1988a), i.e. it may convert low-affinity FMLP receptors to intermediate-affinity receptors.

Our data extend our knowledge by showing that SP primes neutrophils for an increased production of LTB_4 and 5-HETE in response to FMLP plus AA but not A23187.

There are several questions which need to be addressed concerning the role of SP in regulating neutrophil effector function, including specificity and whether the effects are receptor-mediated, and the physiological relevance *in vivo*. To date these questions must remain largely unanswered. The finding that ADCC, but not O_2^- (to any extent) or AA metabolism are stimulated by SP *per se*, argues for a specific effect. We have found that high concentrations of SP are needed to demonstrate its effect on neutrophils and it is uncertain at the moment if neutrophils are exposed to SP concentrations of the magnitude used in our studies *in vivo*. The concentrations needed to demonstrate an *in vitro* effect under our assay conditions are similar to those needed to degranulate mast cells *in vitro* (Shanahan *et al.*, 1985). Nevertheless, there is evidence that, *in vivo*, axon reflexes (which lead to the release of SP) cause a wheal and flare response, part of which is due to secondary release of histamine from skin mast cells (Foreman & Jordon, 1983). These data suggest indirectly that local concentrations of SP (i.e. in the cell micro-environment) may be quite high. Furthermore, a number of factors may modulate the metabolism of SP or the responsiveness of the cell to SP at the site of inflammation *in vivo*, as has been demonstrated for its effects on smooth muscle contraction (Stimler-Gerard, 1987).

In summary, we provide evidence that SP regulates neutrophil effector functions and provide further evidence for a link between the nervous system and the immune response.

ACKNOWLEDGMENTS

The authors wish to thank Dr Angel Lopez for his advice and assistance and Mrs Carolyn Vince for typing the manuscript.

This work was supported by the National Health and Medical Research Council of Australia and the Royal Adelaide Hospital Special Purposes Fund.

REFERENCES

Allen R.A., Traynor A.E., Omann G.M. & Jesaitis A.J. (1988) The chemotactic peptide receptor. A model for future understanding of chemotactic disorders. *Hematol./Oncol. Clinics North America*, **2**, 33.

Atkinson Y.H., Lopez A.F., Marasco W.A., Lucas C.M., Wong G.G., Burns G.F. & Vadas M.A. (1988a). Recombinant human granulocyte-macrophage colony-stimulating factor regulates FMLP receptors on human neutrophils. *Immunology*, **64**, 519.

Atkinson Y.H., Marasco W.A., Lopez A.F. & Vadas M. (1988b) Recombinant human tumor necrosis factor—regulation of N-formyl-methionyl-leucylphenylalanine receptor affinity and function on human neutrophils. *J. clin. Invest.* **81**, 759.

Barnes P.J. (1986) Asthma as an axon reflect. *Lancet*, **i**, 242.

Bar-Shavit Z., Goldman R., Stabinsky Y., Gottlieb P., Fridkin M., Teichberg V.I. & Blumberg S. (1980) Enhancement of phagocytosis—a newly found activity of substance P residing in its N-terminal tetrapeptide sequence. *Biochem. Biophys. Res. Commun.* **94**, 1445.

Dallegri F., Frumento G., Ballestrero A., Goretti R. & Patrone F. (1987). Relationship between antibody-dependent tumour cell lysis and primary granule exocytosis by human neutrophils. *Clin. exp. Immunol.* **70**, 479.

Foreman J. & Jordan C. (1983). Histamine release and vascular changes induced by neuropeptides. *Agents Actions*, **13**, 105.

Hartung H.P., Wolters K. & Toyka K.V. (1986) Substance P: binding properties and studies on cellular responses in guinea-pig macrophages. *J. Immunol.* **136**, 3856.

Holzer P. (1988) Local effect or functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*, **24**, 739.

Jancso N., Jancso-Gabor A. & Szolcsanyi J. (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pre-treatment with capsaicin. *Br. J. Pharmac. Chemother.* **31**, 138.

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.

Lopez A.F., Williamson D.J., Gamble J.R., Begley C.G., Harlan J.M., Klebanoff S.J., Waltersdorph A., Wong G., Clark S.C. & Vadas M. (1986) Recombinant human granulocyte-macrophage colony-stimulating factor stimulates *in vitro* mature human neutrophil and eosinophil function, surface receptor expression and survival. *J clin. Invest.* **78**, 1220.

Lotz M., Carson D.A. & Vaughan J.H. (1987) Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. *Science*, **235**, 893.

Lotz M., Vaughan J.H. & Carson D.A. (1988) Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science*, **241**, 1218.

Lundberg J.M., Saria A., Brodin E., Rosell S. & Folkers K. (1983) A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea-pig. *Proc. natl. Acad. Sci. U.S.A.* **80**, 1120.

McColl S.R., Betts W.H., Murphy G.A. & Cleland L.G. (1986) Determination of 5-lipoxygenase activity in human polymorphonuclear leukocytes using high-performance liquid chromatography. *J. Chromatogr.* **378**, 444.

Marasco W.A., Showell H.J. & Becker E.L. (1981) Substance P binds to the formylpeptide chemotaxis receptor on the rabbit neutrophil. *Biochem. Biophys. Res. Commun.* **99**, 1065.

Omann G.M., Allen R.A., Bokoch G.M., Painter R.G., Traynor A.E. & Sklar L.A. (1987) Signal transduction and cytoskeletal activation in the neutrophil. *Physiol. Rev.* **67**, 285.

Payan D.G. (1985) Receptor mediated mitogenic effects of substance P on cultured smooth muscle cells. *Biochem. Biophys. Res. Commun.* **130**, 104.

Payan D.G., McGillis J.P. & Goetzl E.J. (1986) Neuroimmunology. *Adv. Immunol.* **39**, 299.

Pernow B. (1983) Substance P. *Pharmacol Rev.* **35**, 85.

Serra M.C., Bazzoni F., Della Bianca V., Greskowiak M. & Rossi F. (1988). Activation of human neutrophils by substance P. Effect on oxidative metabolism, exocytosis, cytosolic Ca^{++} concentration and inositol phosphate formation. *J. Immunol.* **141**, 2118.

Shanahan F., Denburg J.A., Fox J., Bienenstock J. & Befus D. (1985) Mast-cell heterogeneity: effects of neoroenteric peptides on histamine release. *J. Immunol.* **135**, 1331.

Stimler-Gerard N.P. (1987) Neutral endopeptidase-like enzyme controls the contractile activity of substance P in guinea-pig lung. *J. clin Invest.* **79**, 1819.

Thureson-Klein A., Hedqvist P., Ohlen A., Raud J. & Lindbom L. (1987) Leukotriene B_4, platelet-activating factor and substance P as mediators of acute inflammation. *Pathol. Immunopathol. Res.* **6**, 190.

Vadas M.A., Nicola N.A. & Metcalf D. (1983) Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. *J. Immunol.* **130**, 795.

Van Gelder B.F. & Slater E.C. (1962) The extinction coefficient of cytochrome c. *Biochim. Biophys. Acta*, **58**, 593.

Wagner F., Fink R., Hart R. & Dancygier H. (1987) Substance P enhances interferon-production by human peripheral blood mononuclear cells. *Regulatory Peptides*, **19**, 355.

Watson S.P. & Downes C.P. (1983) Substance P induces hydrolysis of inositol phospholipids in guinea-pig ileum and rat hypothalamus. *Eur. J. Pharmacol.* **93**, 245.

Weening R.S., Weever R. & Roos D. (1975) Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. *J. Lab. clin. Med.* **85**, 245.