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Substance-P-Mediated Intestinal Inflammation: Inhibitory Effects of CP 96,345 and SMS 201-995

Key Words

Intestine
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Abstract

The proinflammatory peptide substance P (SP) has been shown to be intimately involved in the local inflammatory processes of *Trichinella-spiralis*-induced murine intestinal inflammation. Significant increases in SP, increased myeloperoxidase levels coupled with local morphological deterioration of the jejunum and impaired lymphocyte responses to exogenous SP in vitro have been associated with the model. We have recently determined that the elimination of increased levels of SP via anti-SP antibody therapy can spare the murine gastrointestinal tract much of the pathologies associated with the parasitic infection. Here we further demonstrate that the somatostatin analogue SMS 201-995 as well as the SP receptor antagonist CP 96,345 can effectively decrease the inflammation and lost lymphocyte function seen in the jejunum of *T. spiralis*-infected mice. Again, both intestinal morphology and myeloperoxidase levels were shown to return to normal values upon treatment. The above results suggest that SP is an important modulator of gastrointestinal inflammation.

Introduction

The inflammatory process is a homeostatic mechanism which, using a variety of cell types and soluble mediators, attempts to assault, neutralize and eliminate foreign antigens infiltrating the host. Anatomically, the gastrointestinal (GI) tract is inherently involved in this process due to a continual insult of particulate and soluble antigen into its lumen. It is reasonable to assume therefore that the GI tract possesses the necessary machinery to protect itself and the host from such antigenic encroachment. It has been shown that the GI tract houses a diverse arsenal of inflammatory and immune cells important during inflammation [1-3].

In continuum with the immune and inflammatory cells within the GI tract is the self-contained enteric ner-

vous system. Composed of the myenteric and mucosal plexus, the enteric nervous system, apart from regulating the peristalsis of the GI tract, also has been shown to 'innervate' various immune and inflammatory cells including mast cells and lymphocytes [4-7]. This anatomical association has recently been given functional significance with the findings that nerve-derived products, including substance P (SP), somatostatin (SOM) and vasoactive intestinal polypeptide can effectively mediate immune and inflammatory cell activity. In particular, we and others have shown that SP, vasoactive intestinal polypeptide and SOM can alter immunoglobulin synthesis from murine and

human-derived GI lymphocyte populations, can modify the cytotoxic activity of lymphocytes and can potentiate the production and release of a variety of cytokines from lymphocytes, macrophages and mast

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cells [8-13]. With this in mind, it is reasonable to hypothesize that gut-derived neuropeptides can modulate local immune and inflammatory cell activity.

The presence of such neuropeptides during various inflammatory conditions has been documented. We and others have shown that SP is found in significantly increased amounts in patients suffering from rheumatoid arthritis [14, 15]. Within the GI tract, Mantyh et al. [16] have documented that SP receptors are increased during colitis and Crohn's disease. Complementing this, a variety of studies have documented the changes in SP levels within the GI tract during local inflammatory conditions [17,18].

We have recently characterized the activity of SP in a murine model of intestinal inflammation using the oral instillation of the parasite *Trichinella spiralis*. SP levels, both locally (within the jejunum) and in the serum, are increased by day 4, peak at day 21 and return to normal by day 35 at which time the jejunum has returned to normal morphology. While the levels of SP are increased, the normal response of Peyer's patch and splenic lymphocytes to SP (increased proliferation) is absent; however, as the levels of SP return to baseline, the activity of the lymphocytes, in terms of proliferation, also returns to normal. We have also shown that the manipulation of increased SP levels by the mini-osmotic pump instillation of anti-SP antibodies at the time of infection significantly decreases SP levels, lowers the inflammatory index of gut morphology and restores the reactivity of Peyer's patch lymphocytes to SP [19].

In this report, we have further characterized this process by using the SP receptor antagonist CP 96,345 and a stable analogue of the inhibitory neuropeptide SOM, SMS 201-995, as inhibitors of the *T.-spiralis*-induced intestinal inflammation of the jejunum. CP 96,345 is a non-peptide NK-1-receptor antagonist which has been shown to effectively block the action of exogenous SP in a variety of systems yet its role within the GI tract during inflammatory conditions has not been fully investigated [20].

SOM is a 14-amino-acid neuropeptide which has been shown to possess immunoinhibitory properties as well as anti-inflammatory activities in a variety of studies [3,12]. SOM and its stable analogue SMS 201-995 have been suggested previously to alter the activity of SP [21].

We show here that CP 96,345 and SMS 201-995 are effective exogenous agents in limiting the local gut inflammatory response as measured by histological criteria, lymphocyte activation, endogenous SP levels as well as myeloperoxidase (MPO) activity. While SMS 201-995 is effective in inhibiting the release of SP into the local and

peripheral environment, CP 96,345 effectively blocks the NK-1 receptor; thus, both are able to inhibit the action of SP as a proinflammatory neuropeptide within the gut.

Materials and Methods

Peptides and Chemicals

SP (from Zeneca CRB, Boston, Mass., USA) was resuspended in 0.25 M acetic acid, pH 4.5, aliquoted at 10^{-3} Jff and stored at -70°C. SMS 201-995 (sandostatin octreotide) was commercially obtained (Sandoz, Geneva, Switzerland). CP 96,345 was generously provided by Dr. R. Snider (Pfizer Inc., Groton, Conn., USA). Anti-SP N-terminus monoclonal antibody was a kind gift from Dr. Donald Payan (University of California, San Francisco). 125 I-SP was purchased from Amersham

LS (Canada). Concanavalin A was obtained from Pharmacia (Uppsala, Sweden); RPMI, fetal bovine serum and antibiotics came from Gibco BRL (Canada). ^3H -Thymidine (specific activity 6.7 mmol/Ci) was from NEN (Boston, Mass., USA). All other chemicals were of analytical grade and were purchased from Sigma (St. Louis, Mo., USA).

Animals

Six- to 8-week-old female Balb/c mice were maintained under a 12-hour lighting cycle at 22°C in the McMaster Medical Centre Animal Facility. They were fed normal mouse chow and water ad libitum. At day 0, animals were lightly sedated with ether and gavaged with 150 L3 *T. spiralis* larvae in 150 µl of saline. Muscle larvae (L3) of *T. spiralis* were isolated as described [22].

Immediately after infection, mini-osmotic pumps (Alzet 2002; Alza Co., Palo Alto, Calif., USA) were implanted subcutaneously. The pumps were filled with 200 µl of CP 96,345 or SMS 201-995, which was delivered at a constant rate of approximately 35 and 10 µg/day, respectively, over a 14-day period. Mini-pumps filled with saline were implanted in control infected animals. Noninfected untreated animals were used as additional controls.

All animals were sacrificed on day 21. This was previously determined to be at the peak of inflammation as maximum levels of immunoreactive SP are found in the jejunum and blood [19].

Histology

Alterations in gut morphology were examined as previously described [19]. Briefly, the sections of jejunum were stained with hematoxylin-eosin and alterations in gut morphology were compared to normal by a blinded observer. The degree of inflammation was scored on a scale from 1 (noninflamed) to 10 (inflamed) based on a comparison to uninfected animals, using inflammatory infiltrate, villous atrophy and size, epithelial sloughing, hypertrophy and hyperplasia of smooth muscle as indices. Fifteen animals were examined in each experimental group.

MPO Assay

Two- to 4-cm segments of jejunum were rinsed with PBS (pH 6.0), cut open and the mucosa was isolated, weighed and transferred into homogenization tubes and homogenized. Samples were then frozen on acetone-dry ice, aliquoted to 1 ml and kept at -70°C for up to 2 weeks prior to analysis. Analysis was done by adding 0.1 ml of sample to 2.9 ml of O-dianisidine solution [16.7 mg of O-dianisidine, 90 ml dH₂O, 10 ml PBS (pH 6.0), 50 µl of 1% H₂O₂].

lit

m

Fig. 1. Low-power (x 200; 1), and high-power (x 400; 2) magnifications of normal murine jejunum morphology (A), 21 days after *T. spiralis* infection (C), after treatment with CP 96,345 (D) and SMS 201-995 (B). Note pronounced smooth muscle hyperplasia, villous atrophy, epithelial sloughing, edema and numerous infiltrating cells (C vs. A). Treated animals showed decreased muscle hyperplasia and normalized crypts (B). Villus size is normal; however, epithelial sloughing

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% is still present (D).

Changes in the color versus control were monitored via spectrophotometer analysis, recorded and analyzed automatically and expressed as units of MPO per sample [23].

SP Assay

SP was extracted from 1-cm sections of jejunum as previously described [17,19]. Briefly, the tissue was homogenized using a poly-tron in 1 % acetic acid and centrifuged at 5,000 g for 20 min. Supernatants were collected and frozen at -70°C until further use. Blood was collected in heparinized tubes via cardiac puncture, centrifuged at 1,500g, and the resulting serum was frozen at -70°C and stored up to 2 weeks. SP was detected using a solid-phase, inhibition-type radioimmunoassay as described in detail previously [14,19].

Lymphocyte Proliferation Assay

Lymphocytes were isolated from the spleen of infected animals as well as controls as was previously described [19]. Spleens were teased apart in RPMI media containing 200 mM L-glutamine, 10mmol Hepes buffer, 40 ug/ml gentamicin, 100 U/ml of penicillin, 100 rag/ ml of streptomycin and 10% fetal bovine serum. Cells were washed 3 times and plated at a concentration of 2 x 10⁵ cells/well in 96-well flat-bottom plates (Corning Co., Oakville, Ont, Canada) in 200 u_l of complete RPMI with 2 ug/ml concanavalin A and with or without 10⁻⁸M of SP. Cells were incubated in a humidified incubator at 37°C in 5% CO₂. After 48h, 1 uCi of ³H-thymidine (specific activity 6.7 mmol/Ci) was added to each well, and cells were incubated for additional 24 h. Cells were harvested, and ³H-thymidine uptake was quantitated using scintillation counting [19].

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Table 1. Degree of jejunal inflammation

Animals	Treatment	Score
Noninfected	none	1.3 (0.1)
Infected	none	7.2(2.1) ^a
Infected	CP 96,345	4.4 (0.6) ^b
Infected	SMS 201-995	3.8 (1.2) ^b

Values represent a score of inflammation on a scale from 1 (noninflamed) to 10 (severely inflamed), with standard deviations in parentheses, and are from at least 15 animals per treatment group in a total of 4 experiments.

^a $p < 0.01$: statistically significant difference as compared to noninfected animals; ^b $p < 0.05$: statistically significant reduction as compared to infected, untreated animals.

$P < 0.05$

■ -noninfected ■ -*T. spiralis*

CP 96,345 ^a SMS 201-995

Fig. 2. MPO levels in the gut tissue of untreated and treated mice on day 21 after infection with *T. spiralis*. Values are given in units of MPO per milligram of wet tissue. Error bars represent standard deviations.

Statistics

Data were compared using analysis of variance. A p value < 0.05 was deemed significant. Error bars on figures represent the standard deviation measured at each point.

Results

Histological analysis showed that jejunum isolated from infected animals had pronounced smooth muscle hyperplasia, villus atrophy and severe epithelial sloughing (fig. 1C) as compared to noninfected mice (fig. 1A). Animals treated in vivo with CP 96,345 or SMS 201-995 had significant decreases in inflammatory responses in the gut (table 1). Treatment with SMS 201-995 normalized epithelial cells and slightly decreased smooth muscle hypertrophy and crypt hyperplasia (fig. 1B). As shown in figure 1D, the jejunum of mice treated with CP 96,345 had reduced smooth muscle hypertrophy and hyperplasia, had normalized villous size and architecture, but epithelial sloughing was still present.

The MPO activity assay was employed to characterize the level of inflammatory cell infiltrate into the gut. The MPO activity in the jejunum of *T. spiralis*-infected animals (fig. 2) was significantly increased (from 0.21 ± 0.06 to 1.62 ± 0.25 units/mg of tissue). When animals were treated with CP 96,345, *T. spiralis* infection did not cause as high an inflammatory response as in untreated infected animals (0.75 ± 0.11 units/mg tissue). Subjection of the animals to SMS 201-995 resulted in an even more pronounced decrease in MPO activity (0.49 ± 0.1).

Figure 3 shows that inflammation provoked by *T. spiralis* caused a significant increase in SP in the gut (fig. 3A; from 0.376 ± 0.121 to 10.63 ± 3.45 pg/mg of tissue). In animals treated with CP 96,345, the level of SP was 3 times higher (29.24 ± 6.28 pg/mg of tissue) than in untreated infected mice. Treatment with SMS 201-995 had an inhibitory effect. The level of SP in SMS-201-995-treated animals was significantly lower (6.09 ± 1.46 pg/ml of tissue) than in both CP-96,345-treated and untreated infected mice. In blood (fig. 3B) the effects of inflammation and treatment were less pronounced but had the same patterns. *T. spiralis* infection caused a slight but not statistically significant increase in serum SP levels (from 410.0 ± 25.5 to 504.3 ± 161.2 pg/ml; fig. 3B). Treatment with CP 96,345 significantly increased (730.2 ± 131.8 pg/ml) serum levels of SP, while SMS 201-995 decreased (176.5 ± 85.4 pg/ml) serum SP levels.

In noninfected animals, exogenous SP (10^{-8} M) significantly increased proliferation of splenic mononuclear cells (fig. 4) from $2,875 \pm 753$ cpm to $5,072 \pm 779$ cpm. In animals infected with *T. spiralis*, the proliferative response to SP was indicatively decreased ($2,000 \pm 225$ cpm) compared to noninfected controls. Treatment with CP 96,345 did not restore proliferative response of splenocytes to SP but increased ³H-thymidine incorporation to $2,688 \pm 343$ cpm. When animals received SMS 201-995, the ability of splenocytes to respond to SP stimulation was fully restored ($6,332 \pm 597$ cpm).

P<0.01

Fig. 4. Effect of CP 96,345 and SMS 201-995 treatment on splenocyte proliferation. The dotted line shows the level ($\pm$ SD) of ³H-thymidine incorporation in cells from noninfected mice cultured in the presence of 2 ng/ml of concanavalin A. Bars show the amount of ³H-thymidine incorporated by the cells incubated with 2 μ g/ml of concanavalin A and 10⁻⁸M of SP. Values are given in cpm/2 x 10⁵ cells. Error bars represent standard deviations.

300

150 -

Fig. 3. The levels of immunoreactive SP in jejunum (A) and (B) on day 21 after infection and after treatment with CP 96,345 or SMS 201-995. Error bars represent standard deviations.

Discussion

The 11-amino-acid neuropeptide SP is now recognized as an immunomodulating and inflammatory peptide. Its intimate involvement in various inflammatory conditions, including rheumatoid arthritis [14, 15, 24-26], asthma [27-29] and inflammatory bowel disease [16, 20, 30], has been established. At the cellular level, SP has been shown to activate numerous cell types, including both immune (lymphocytes) and inflammatory (neutrophils, macrophages) and stromal (fibroblasts, endothelial) cells all with a resulting net proinflammatory signal [3, 4, 8, 13, 31, 32]. However, it is unknown if SP is an inducer of inflammation or if its levels rise as a consequence of the inflammatory process. Until very recently, information regarding the connection between inflammatory conditions and the cellular activation by SP has been limited. Using an acknowledged murine model of intestinal inflammation (jejunal *T. spiralis* infection), we have shown that the presence and activity of SP is paramount in driving many of the inflammatory processes observed, both locally and systemically [19]. Previous to this study we demonstrated that by blocking the action of SP in vivo (with anti-SP antibodies) we could spare the intestine from the major insult of the *T. spiralis* infection [19]. In this report, we have analyzed the effect of the SP receptor antagonist CP 96,345 and the stable SOM agonist SMS 201-995 within this model. Our first observation illustrated that in vivo treatment with SMS 201-995 could significantly reduce the degree of jejunal inflammation. While this is a novel phenomenon within the inflamed gut, SOM and its analogues have been shown to possess anti-inflammatory properties in other systems [26]. While the mechanism for the action of SMS 201-995 in other systems is only speculative, we feel that the ability of SOM and its analogues to inhibit the release of SP is critical to its action. In our *T. spiralis* model, we have shown that SMS 201-995 does inhibit SP production from isolated jejunum and serum of infected animals. Others have

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shown that SOM can inhibit the activity of SP in various systems [21] maintaining this observation as a viable hypothesis. Further, we were able to demonstrate that SMS 201-995 could effectively inhibit the release of MPO from infiltrating cells. The ability of SP to induce neutrophil chemotaxis has been well documented [32]. We and others have shown that this may be accomplished by the direct stimulation of SP receptors or via the regulation of production of various cytokines by various cell types when stimulated with SP in vitro [33-35]. SOM and its agonist have been shown to affect inflammatory cell chemotaxis in vitro [36]. We also have preliminary evidence that SOM can inhibit interleukin-8 production from various cell types in vitro [Agro, Stanis, unpubl. observations]. Therefore, it is plausible to suggest that SMS 201-995 may act via the cytokine network to inhibit the induction of MPO by infiltrating neutrophils; however, a direct effect on these cells at the signal transduction level cannot be ignored.

We have been able to couple the activation of lymphocytes by various neuropeptides with specific surface receptors for these molecules on the lymphocyte surface [34, 35]. Lymphocyte proliferation is a somewhat crude measure of immune cell function after stimulation. We have shown previously that lymphocytes isolated from normal murine spleen and Peyer's patches are stimulated into S phase and further replication by SP is inhibited by SOM and its analogues [8, 12]. Lymphocytes isolated from conditions of inflammation (rheumatoid arthritis, *T. spiralis* model) are refractory to exogenous stimulation with SP. We have shown that this is due to a desensitization of SP receptors on lymphocytes as a result of the significant increase in SP levels seen in these conditions [14]. During peak inflammation, we show here that SMS 201-995 treatment in vivo can restore the responsiveness of lymphocytes to SP. This is most likely due to the decrease in SP levels induced by SMS 201-995 and the resulting re-expression of SP receptors able to respond to significantly lower levels of SP. We are presently analyzing NK-1 receptors to verify this hypothesis. The lymphocyte response to the mitogen concanavalin A is significantly inhibited as compared to

normal uninfected animals. During the inflammatory reaction, glucocorticoids, catecholamines, cytokines and various other mediators (all of which could affect lymphocyte proliferation) are elevated in the blood. SP seems to be one of many such mediators. Therefore, it is no surprise that upon inhibition of SP lymphocyte proliferation could only be partially restored.

CP 96,345 is a nonpeptide SP receptor antagonist which preferentially blocks NK-1 receptors impairing the

target cells' ability to respond to SP [37]. Although it has some non-specific effects (e.g. on calcium channels) [38], it has been shown to block many of the properties of SP in various systems and cell types [39]. The in vivo instillation of CP 96,345 had no inhibitory effect on SP levels either in the serum or the jejunum of infected mice. In fact, treatment with CP 96,345 actually enhanced the measured level of SP found within the tissue of *T. spiralis*-infected animals. With SP receptor occupancy being altered by the presence of CP 96,345, the higher level of SP seen within jejunal extracts is explainable. Upon analysis of a more distal tissue to the inflammation (the blood), it is apparent that there also exists a difference between SP levels in *T. spiralis*-infected animals and those treated in vivo with CP 96,345.

Of interest was the finding that in vivo treatment with CP 96,345 was able to reduce the jejunal pathology and MPO activity versus untreated animals. As far as we know, this too is a novel finding. A direct effect of CP 96,345 on plasma extravasation has recently been documented [40]. These findings coupled with the reduced pathologies seen in CP-96,345-treated animals further suggest that inhibiting the SP receptor or inhibiting the synthesis of SP can act as a protective device within the inflamed gut.

In vivo administration of CP 96,345 was not able to restore the responsiveness of lymphocytes to exogenous SP. This suggests that the inability of CP 96,345 to inhibit the release/production of SP is the reason for maintained lymphocyte unresponsiveness or that immune cell activation by SP is mediated by non-NK-1 receptors [41].

The above results, coupled with our previous observations, strongly suggest that SP is an important mediator of jejunal inflammation in the *T. spiralis* model of murine intestinal inflammation. Limiting the overproduction of SP by the SOM analogue SMS 201-995 combined with the inhibition of the SP receptor by the nonpeptide receptor antagonist CP 96,345 may be useful tools in the management of many in vivo models of intestinal pathology. We are currently analyzing the effect of various combinations of both SP inhibitors in our model with the hope that we will eventually be able to alter the progression of inflammation within the human system.

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