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THE ROLE OF TACHYKININS ON BACTERIAL INFECTIONS

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TABLE OF CONTENTS

1. ABSTRACT

Tachykinins represent a family of neuropeptides sharing similar C-terminus sequences, but exhibiting preferential binding to one of three receptors called neurokinin receptors (NK-R). While known for its role in contracting smooth muscle or acting as a pain signal neurotransmitter, substance P (SP) and other tachykinins can directly influence immune responses. Studies from the early 1980s revealed that human lymphocytes bore NK-R, but it remains unclear, even to-date, why such receptors are expressed on leukocytes. Nerve tracing studies have provided some speculation that the nervous system can assist the immune system in stimulating an immune response dependent upon which neuropeptide-bearing fibers infiltrate specific lymphoid structures. Such observations have important implications for regulating mucosal responses given that tachykinin-bearing nerve fibers extensively innervate the gut, and SP concentrations in the gut are second only to the brain. Such evidence suggests that SP and related neuropeptides may be important in controlling bacterial infections of the gut. This is shown by blocking SP action in which mice show increased susceptibility to *Salmonella* infections since induction of IFN-gamma is significantly reduced. In addition, the absence or its presence of SP's or the newly discovered lymphocyte-derived neurokinin called hemokinin's action can modify host IgA responses. Thus, tachykinins introduce new circuits to immune regulation suggesting that these neuropeptides exhibit cytokine- and chemokine-like action.

2. INTRODUCTION

Neurotransmitters, neuropeptides, and neuroendocrine hormones are often described in the context of their neural functions, often without much consideration of possible immune attributes. While it is already difficult to image the layers of immune regulation without having to consider neural pathways, neural tracing studies suggest that indeed the nervous system can impact immunity. In addition, products of the nervous system are also produced by leukocytes suggesting that these molecules have extraneuronal functions. This review is a discussion of how substance P (SP) can influence adaptive immunity, in particular, in the context of bacterial infections. The relevance of neural intervention probably has its greatest influence in the mucosa because of the density of its innervation by the sensory neurons, particularly the peripheral nerve fibers (peptidergic fibers) containing the neuropeptides (stored in secretory vesicles).

3. THE ROLE OF TACHYKININS IN BACTERIAL INFECTIONS

3.1. The players

Recognized for its ability to contract ileum smooth muscle cells and to act as a pain neurotransmitter, many of the earlier studies of substance P (SP) focused on its physiological capabilities (1, 2). This 11 amino acid neuropeptide, SP (Table 1), is a product of the sensory ganglion cells, and it is transported to peripheral sites where it is stored and released on noxious stimulation (1). SP belongs to a family of related peptides called tachykinins in which each member bears the C-terminus amino acid sequence, Phe-X-Gly-Leu-Met-NH_2, with X- being a branched aliphatic or aromatic amino acid, and the C-terminal amino acid is amidated (Table 1). Not surprisingly, these sequences are highly conserved among mammals (bovine, human, mouse, and rat sequences). Two other related tachykinins, substance K (neurokinin A) and neuromedin K (neurokinin B) also belong to this family of neuropeptides. Most recently, a fourth neuropeptide has been discovered (3, 4) called hemokinin, which is believed to be responsible for many of the activities associated with SP function on the immune system.

These neuropeptides are encoded by three mammalian tachykinin genes: preprotachykinin A (PPT-A) generates SP and neurokinin A, and by alternate RNA splicing, four distinct SP-encoding mRNAs are produced and designated as alpha, beta, gamma, and delta forms of

Table 1. Tachykinin Amino Acid Sequences

Gene	Translated Products	Amino Acid Sequences
preprotachykinin A	substance P (SP)	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	substance K (neurokinin A)	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
preprotachykinin B	neurokinin B (neurokinin B)	Asp-Met-His-Phe-Val-Gly-Leu-Met-NH_2
preprotachykinin C	hemokinin (HK)	Arg-Ser-Arg-Thr-Arg-Gln-Phe-Tyr-Gly-Leu-Met-NH_2

PPT-A; preprotachykinin B (PPT-B) generates neurokinin B; and preprotachykinin C (PPT-C) generates hemokinin (3, 4).

However, neuronally-derived SP may not be the only source of this neuropeptide during immune responses. Following activation, a variety of leukocytes have the potential to express neuropeptides. PPT-A mRNA expression has been detected in cultured macrophages, (5-10), dendritic cells (11), lymphocytes (10-12), and neutrophils (13) following stimulation. In addition, PPT-C mRNA expression has been reported in B lymphocytes (3). Fewer reports have quantified the levels of SP peptide that can be derived from macrophages (5, 7, 14), dendritic cells (11), lymphocytes (10) and neutrophils (13). Collectively, these studies clearly demonstrate that stimulated leukocytes can express PPT mRNA and the products of PPT genes, albeit at concentrations that are significantly less than those reported for neuronal-derived tachykinins.

3.2. The receptors

Much of the earlier studies focused on identifying the receptor for tachykinins. The limitation of these earlier studies was requisite demonstration that lymphocytes bore receptors for these neuropeptides since the sequence for the tachykinin receptors had not been determined until the late '80s and early '90s (15-17). Prior to such findings, receptor binding studies and ligand rank displacement studies were required to verify that the Kd was identical to the neuronal receptor. Ligand binding studies performed with nonlymphoid receptors revealed that there were three receptors functionally distinct by their preference in binding to SP, neurokinin A, or neurokinin B (1, 2, 18). Thus, the tachykinin or neurokinin receptor (NK-R) family was found to be composed of three closely related G-protein coupled seven transmembrane receptors sharing sequence homology, but differing in ligand specificity (2, 18). The neuronal SP receptor or NK-1R shows the greatest affinity for SP (2, 18) and hemokinin (19, 20). This receptor is identical to the NK-1R expressed by leukocytes. In fact, macrophages (7, 21-26), dendritic cells (25, 27), T lymphocytes (12, 28-33) and B lymphocytes (6, 34-39) express NK-1R.

Much of the earlier descriptions for the NK-1R were derived from studies evaluating the human T cell lymphoblast line, IM-9 (34, 40-42). Subsequent studies showed that in humans the NK-1R may serve as a marker to distinguish human mucosal B and T cells from peripheral lymphocytes (24, 43). SP and neurokinin A also appear to be chemotactic for human lymphocytes rather than monocytes (44), suggesting the expression of NK-1R and NK-2R is functional.

3.3. Anatomical evidence for SP's role in immunity

It is important to note that outside the brain, SP is found in greatest concentrations in the gut (1, 2), and this neuropeptide can also be found in high levels in the lungs (45). This presence of SP suggests that SP may contribute to the regulation of immune function in gut-associated lymphoid tissues (GALT) and bronchus-associated lymphoid tissue (BALT). Bearing this in mind, let us consider the components of the mucosal immune system. Mucosal tissues have two major components: inductive sites and effector sites. Inductive sites are where antigens are first encountered and processed and where initial induction of immune and memory B and T cells occurs. In the gut, the Peyer's patches represent the inductive sites for the GALT (46, 47), and the nasal-associated lymphoid tissue (NALT; 48-50) has recently been identified as the Peyer's patch equivalent for the upper respiratory tract. For the GALT and upper respiratory tract, the Peyer's patches and NALT, respectively, can be functionally and anatomically separated into three distinctive areas: 1) the dome with a unique lymphoepithelium; 2) the B cell follicles, which usually contain one or more germinal centers; and 3) the perifollicular or T cell dependent area (51). The dome region is covered by an epithelium referred to as follicle-associated epithelium (FAE). Within the FAE, there is a differentiated epithelial cell subset referred to as M cells (52-54). M cells can sample soluble proteins (53, 55-57) or viruses, such as reovirus types 1 and 3 (58), HIV (59, 60), poliovirus (61), and rotaviruses (62). In fact, the majority of the enterobacteria family, i.e., *Salmonella*, *Escherichia coli*, *Vibrio cholerae*, and *Shigella*, are transported through M cells (56). Consequently, many of the bacterial and viral pathogens actually exploit the M cells as a means of infecting the host. This is where antigen and vaccine uptake into the inductive tissues leads to effective presentation to MHC class II restricted $CD4^+$ Th cells, and for intracellularly processed and class I associated peptides, it leads to $CD8^+$ lymphocytes and CTL precursors, resulting in local immune protection. Furthermore, B cells in inductive tissues respond to antigen and undergo expansion and memory cell formation through the help of Th cells, and they disseminate to mucosal effector tissues such as the diffuse lamina propria (LP) regions of the small and large intestine and nasal passages. After settling into these effector tissues, the B cells may undergo clonal expansion and differentiation into IgA plasma cells upon re-exposure to Ag and help from Th-derived cytokines.

Some of the first studies considering the possibility of potential cross-talk between a product of the central nervous system (CNS) and the immune system began with two studies.

O'Dorisio *et al*. (63) showed a non-neuronal source for vasoactive intestinal peptide (VIP) in neutrophils, but VIP could activate lymphocyte adenylate cyclase (64). Payan *et al*. (65) showed that SP could

enhance human T cell proliferative responses, and that human lymphocytes expressed the SP receptor (34, 40), whereas Beed *et al.* (66) showed that the receptor for the neuropeptide, VIP, was expressed by the human MOLT 4b T cell lymphoblasts. Such observations led to the question of why mononuclear cells would need to express neuropeptide receptors. Neuronal tracing studies provided part of the answer showing direct interaction between leukocytes with nerve cells. This was shown in a pair of studies where direct evidence between gut mast cells and SP-containing nerve fibers (67, 68). In the Peyer's patches, SP-containing nerve fibers infiltrate T cell zones to contact macrophages avoiding B cell areas. In contrast, intestinal lamina propria IgA plasma cells were found in densely innervated areas, suggesting that these cells perhaps may be more likely to be influenced by neuropeptides (67, 69, 70). Evaluation of mesenteric lymph nodes showed that SP-containing nerves were sparse and found to be associated with 5 - 10% of the arterioles and venules in the medulla adjoining the T cell region and in the capsule (71). In the same study, SP receptor binding sites were examined by quantitative receptor autoradiography. SP-containing fibers were also found in the BALT. In the rat BALT, SP-containing fibers innervated the subepithelial zone (72). In the human tonsil, SP-containing fibers were found in the perivascular plexus with low level expression in the interfollicular areas and adjacent to T cells and macrophages (73). The association of SP containing neurons with lymphoid organs is phylogenetically conserved, since the Bursa of Fabricus in birds contains SP fibers that contact B lymphocytes (74). Thus, we have described the presence of peptidergic nerve fibers in close approximation with lymphocytes. This evidence suggests that indeed mononuclear cells are modified by neuropeptides and the presence of neuropeptide receptor on lymphoid cell surfaces corroborate this point. To address the function of these neuropeptides on antigen-driven immune responses, and the role of these neuropeptide receptors expression on lymphocytes and macrophages, the following studies describe the immune modulation on B cells, T cells, and antigen-presenting cells.

3.4. Tachykinins and adaptive immunity

Some of the initial studies to examine SP's function evaluated whether B cells could be induced to secrete Ig. It was found that SP enhanced concanavalin A activated splenic, mesenteric lymph node, and Peyer's patch mononuclear cells resulting in 70%, 40%, and 300% increases, respectively, in IgA production (75). To a lesser extent, IgM levels were altered significantly by 20 to 40%, but not IgG levels. Given the context of anatomical data, this observation suggested that SP may preferentially stimulate IgA secretion, or alternatively it may indicate that SP is an IgA switching factor. A more recent study using human T and B lymphocyte co-cultures showed that SP, neurokinin A, and neurokinin B could augment IgA and IgG_4 secretion in IL-5 or TGF-beta co-stimulated cultures (76). These collective *in vitro* studies suggest that B lymphocytes can be directly stimulated by tackykinins, or they require the presence of an additional $NK\text{-}1R^+$ cell subset that is responsive to tachykinins which in turn augment B cell responses.

To delineate between these two possibilities and to study whether SP can act as a B cell differentiation factor (6, 36, 37), cloned IgM^+ and IgA^+ B lymphoma cell lines were tested. Radiolabelled binding studies revealed that these CH12.LX subclones were $NK\text{-}1R^+$ with the appropriate Kd. Initial tests of whether SP could directly augment antibody production of CH12.LX.C4.4F10 (IgA^+) B cells showed only modest enhancement, whereas similar stimulation on CH12.LX.C4.5F5 (IgM^+) B cells showed no augmentation. However, as previous studies suggested, SP's capability to augment Ig synthesis may be tied to a second signal. The addition of physiological concentrations of SP to LPS-co-stimulated CH12.LX.C4.5F5 cells enhanced IgM production or to LPS-co-stimulated CH12.LX.C4.4F10 cells enhanced IgA production. In a similar fashion, purified splenic (36) and Peyer's patch B cells (38) required a co-stimulation signal in order to effect Ig augmentation. Physiological SP concentrations augmented LPS-co-stimulated splenic B cell IgM and IgG3 production (36), and augmented IL-6-co-stimulated Peyer's patch IgA and IgG production (38). Collectively, these studies demonstrate that SP can directly affect B cells in the absence of antigen-presenting cells or T cells, and the observed Ig augmentations occurred with physiologically relevant SP concentrations supporting the notion that the B cell NK-1R is functional. This requisite for a co-stimulatory signal implies that under resting conditions SP has a minimal effect upon B cells. This is further supported by the observation that SP-containing nerve fibers fail to infiltrate into the PP B cell zones, suggesting that repeated or direct stimulation of PP B cell subsets can be avoided. This aversion may also represent an additional means of regulating B cell activation, and possibly during an inflammatory response, innervation into the B cell zones may occur resulting in their activation. Alternatively, SP may also direct its action on T cells and accessory cells (see below), or expression of the NK-1R is more likely for mediating signaling by the newly described NK-1R agonist, the tachykinin, hemokinin (19, 20). It was recently shown that high concentrations of hemokinin stimulated the proliferation of IL-7 expanded B lymphocytes (3). Alternatively, it is speculated that hemokinin may act as an autocrine factor promoting the survival of B cell precursors in the bone marrow (3).

The *in vivo* relevance of the above described *in vitro* studies were shown by Helme *et al.* (77). Using the neurotoxin, capsaicin, which destroys unmyelinated sensory neurons present in peripheral tissues (78) and thereby depleting peripheral SP (and other neuropeptide) levels, neonatal rats were treated, allowed to mature, and then immunized with sheep red blood cells (SRBC). As a result, greater than 80% reduction in IgM and IgG plaque-forming cell responses by popliteal lymph nodes were observed when compared to plaque-forming cell responses in untreated SRBC-immunized rats. These results clearly demonstrate the importance of the nervous system, and in this case, the relevance of neuropeptides for developing antibody responses. This lack of antigen responsiveness

exhibited by capsaicin-pretreated rats was reversible upon co-administration of SP with SRBC. Likewise, rats treated with the SP antagonist, Spantide, during antigen priming caused similar reduction in antibody responses (79). Thus, these studies show that SP can impact antibody responses to particulate antigens.

Aside from the early functional studies showing that SP supports T cell proliferation (65, 75), implications that T lymphocytes can express NK-1R, NK1 mRNA expression by cultured murine (29) and human T cells (14) or T cell lines have been reported. The functionality of T cell NK-1R was demonstrated by co-cultures with SP-producing dendritic cells (11). Interestingly, NK-1R mRNA expression was observed in intraepithelial and lamina propria T lymphocytes, but not in splenic T cells (12).

3.5. Tachykinins and *Salmonella*

Since SP is present at nanomolar concentrations in the gut, this is suggestive that SP contributes to the regulation of immune function in gut. Thus, a mucosal infection model would seem to be the most likely candidate to study the role of SP upon immunity. In this context, few studies have examined the relevance of SP upon bacterial infections. The majority of such studies focused on immune responses subsequent to wild-type or attenuated *Salmonella* infections. One reason to study *Salmonella* infections is because *Salmonella* enters through the gut mucosa where it survives as an intracellular pathogen of macrophages and dendritic cells. Successful clearance of this pathogen requires IL-12 induced IFN-gamma production, which amplifies macrophage and dendritic cell activation, resulting in *Salmonella*'s elimination (80). As such, using the murine salmonellosis model was investigated to determine the importance of SP upon macrophage activation (23).

From these studies, it has become evident that NK-1R expression by macrophages and dendritic cells has an important role for resolving *Salmonella* infections, at least at the initiation of the host response. The rationale for hypothesizing that SP might play an important role in antigen-presenting cells (APC) activation and destruction of *Salmonella* is derived from a series of *in vitro* studies examining the impact of SP upon macrophage function and the demonstration that NK-1R can be induced by these cells (7, 21, 23, 25, 81-84). Cultured macrophages exposed to *Salmonella* were found to rapidly upregulate expression of NK-1R (23). Such a response had added significance since it was already established that SP could augment the production of reactive oxygen intermediates by these cells (21). Thus, an upregulation of NK-1R expression by *Salmonella* might significantly increase the SP-mediated macrophage response, directly killing the bacteria, as was suggested in earlier studies showing that SP could enhance the production of the proinflammatory cytokines, IL-1, IL-6, and TNF-alpha by macrophages (81). Together with the results showing that SP could enhance LPS-induced production of bioactive IL-12p70 (82) suggests that neurokinins maybe important proinflammatory inducers. Moreover, these *in vitro* studies suggests that SP might augment the ability of macrophages to kill *Salmonella*, as well as mechanisms which would enhance the cell mediated immune response via production of monokines.

Using the information derived from the above described studies, *in vivo* evaluations were performed to study the relevance of SP and its receptor in mucosal immune responses against *Salmonella*. Interestingly, subsequent to oral infection with *Salmonella*, rapid and dramatic upregulation of the mRNAs encoding SP (85) and its receptor (23) was observed in mucosal tissues. This result suggested that SP and its receptor were involved in the initiation of the response against this pathogen. To directly address this possibility, mice were pretreated with the potent SP antagonist, Spantide II, prior to oral challenge with *Salmonella*. Mice pretreated with this SP antagonist could not resist the bacterial infection as well as control mice pretreated with an irrelevant peptide (23). Treatment with the antagonist caused no apparent alterations in gut function aside from a reduction in IL-12p40 mRNA expression *in vivo* following oral inoculation with *Salmonella*. Therefore, *in vivo* antagonism of SP/NK-1R interactions resulted in surprising and dramatic reductions in the resistance against the intracellular pathogen, *Salmonella*.

To further address the role of SP contribution to S-IgA responses, NK-1R$^{-/-}$ mice were orally immunized with an attenuated *Salmonella* construct expressing colonization factor antigen I (CFA/I). This vaccine construct has been shown to elicit a biphasic Th cell response (86) supported by an early robust IL-4- and IL-5-producing CD4^{+} T cells. When such construct was used to orally immunize NK-1R$^{-/-}$ mice, a significant increase in antigen-specific S-IgA titers were obtained (87). Surprisingly, no significant differences in IFN-gamma production were observed between NK-1R$^{+/+}$ and NK-1R$^{-/-}$ mice, but increased production to IL-6 was obtained. This evidence suggests minimally, that some intracellular infections are resolvable in the absence of NK-1R function, perhaps via increases in S-IgA responses.

4. SUMMARY AND PERSPECTIVES

In this chapter, we have provided experimental evidence for the role of neurokinins in bacterial infections. Having shown that SP can play a role in augmenting both innate and adaptive immune arms, future studies showing the intracellular mechanisms involved become increasingly significant. In view of these observations, it remains to be resolved why neurokinins can be derived from other than neuronal cells since SP containing neurons have been localized to many lymphoid tissues. As past studies have shown, leukocytes can synthesize SP (5) and VIP (63, 91-94). One aspect of the studies addressing leukocyte-derived neuropeptides that has failed to gather consideration is the question why these neuropeptides are produced by leukocytes. Speculatively, there are several possible explanations for the importance of leukocyte-derived tachykinins. First, it is highly likely that the stimuli which induce neuronal production and secretion of tachykinins will be significantly different from those stimuli which would evoke SP secretion by leukocytes. Therefore, the

ability of leukocytes to produce SP during immune responses might reflect a need to respond to a diverse array of stimuli. Alternatively, this may represent an amplification mechanism to retain proinflammatory responses as observed in the *Salmonella* infection studies. Third, it is likely that leukocyte-derived neuropeptide production will allow such peptides to be produced in areas of limited innervation by peptidergic neurons. This would permit tachykinins to contribute to the development of immune responses in the absence of neuronally derived peptide. While intricate studies have shown the production of tachykinins by leukocytes, studies describing their *in vivo* relevance have been minimal. One possibility for their production is that low level production of neuropeptides may represent a mechanism for maintaining the expression of their respective receptors on the leukocyte cell surface. In the case of macrophage-derived SP (5), the proponents offered that macrophages produced SP in a paracrine/autocrine fashion to regulate cytokine production. Thus, if leukocyte synthesized neuropeptides can affect their own function, then we must consider them functionally similar to cytokines. It may be important for them not to function at neuronal or endocrine sites; they may instead exhibit properties not previously considered. Consequently, leukocyte-derived neuropeptides introduce a novel regulatory circuit to immune regulation, possibly behaving as cytokines do. Such an additional regulatory pathway may also have its own mode of neuropeptide release. Previous studies have shown the sensitivity of neural elements to cytokine stimulation to induce the release of neuropeptides (95, 96), but these same cytokines may not affect leukocyte-derived neuropeptide release.

As suggested by some of the previously described studies, the impact by SP will most likely occur in a localized area as opposed to inducing a systemic effect, but may very well impact the development of memory responses. Being inherently mobile, this allows leukocytes greater opportunities for neural-immune interactions and suggests that leukocytes may be transiently innervated. Evidence to this effect has been shown by Felten *et al.* (88), in which ultra-structural studies with rat spleens showed synaptic-like contacts between sympathetic nerve fibers and lymphocytes. This close approximation between lymphocytes and nerve fibers (67, 89, 90), especially mucosal sites in the gut, provides physical evidence for cross-communication between the immune and nervous systems. Such physical evidence, combined with shared usage of molecules, e.g., neurotransmitters, neuropeptides, neuroendocrine hormones, suggests that the nervous system does impact lymphocytes, whereas cytokines can affect neural function. Moreover, to facilitate this cross-talk, one of the intriguing observations to-date is the expression of neuropeptide and neuroendocrine hormone receptors on leukocytes. In particular, it is interesting to note the varied expression of NK-R by various leukocytes.

There is still much to learn about the mechanisms utilized by the nervous system to regulate immune function in the MALT. We are beginning to learn about the ability of lymphocytes and macrophages to respond to tachykinins. Subsequent studies should provide insight into the modulation of NK-R on leukocytes and, in particular, lead to an understanding of the regulatory mechanisms and events responsible for the expression of NK-R. This can be especially significant in mucosal tissues where the presence of tachykinins is greatly associated with leukocytes. The consequence of understanding the relationship between the nervous system and the immune system will provide a basis for future treatment of bacterial infections. Thus, with the development of cDNA probes and monoclonal antibodies to NK-R, the regulation of these receptors on lymphoid cells and macrophages can be addressed readily, providing a functional understanding of the neural-immune network.

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