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Effect of zymosan-induced peritonitis on the expression of substance P in primary sensory neurons and spinal nerve processes

Thesis submitted in partial fulfillment of Honors

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Abstract:

Macrophages and other cells of the innate immune system recognize foreign particles that could be potentially dangerous and respond by initiating an inflammatory response. The biologically active chemical mediators of this response called pro-inflammatory cytokines are produced in various myeloid derived immune cells and can affect other cells of the body. Interleukin-1 β , a pro-inflammatory cytokine, has been shown to have direct effects on dorsal root ganglion (DRG) cell bodies including the upregulation and direct release of a nociceptive neurotransmitter called substance P (SP). Using a zymosan-induced model of systemic inflammation, we hypothesized that murine DRG neurons and the nerve processes associated with them in the dorsal horn of the spinal cord (SC) at the L1 level will show an upregulation of SP expression in response to inflammation in the peritoneum. Experimental mice were treated with a zymosan suspension (500mg/kg, intraperitoneal injection), and control mice received sterile filtered solution (intraperitoneal injection). Both DRG and SC specimens were collected after *in situ* fixation and subjected to immunofluorescence staining to label SP. Using confocal microscopy, fluorescence microscopy, and image analysis software this expression of SP was quantified and compared. In both tissue specimen groups, an increase in SP expression was discovered in zymosan treated mice. The exact cause of this increase was not specifically determined in this experiment. This experiment provided valuable insight about how a systemic inflammatory response can affect sensory nerve function. Successful methods for further experimentation were identified and information about the zymosan model of inflammation was obtained.

Introduction:

The innate immune system is the first line of defense against foreign pathogens. This complex system recognizes potentially dangerous materials entering the body and unleashes an arsenal of nonspecific defenses to combat the intruder in order to keep the host organism free from dangerous infection. In combination with special types of physical barriers including the skin and mucous membranes, a line of cells produced in bone marrow called myeloid cells have specific functions that allow them to deal with foreign and potentially dangerous organisms or toxins. Macrophages have long been known to play an essential role in the immune and inflammation reactions of the tissue to foreign organisms. Macrophages are important in both ingestion of foreign entities and secretion of biologically active chemicals that further the immune response (Johnston, 1988).

The activation of macrophages to secrete these biologically active chemicals relies on the recognition of a molecule as non-self. This is accomplished in part by a class of receptors called toll-like receptors (TLRs). These receptors are expressed as both membrane receptors and intracellular receptors that can recognize molecules of foreign origins by binding to domains common to foreign classes of organisms (Moresco, 2011). The common macromolecules recognized by these receptors are known as pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs), and the receptors that recognize these molecules are known as pattern recognition receptors (PRRs). TLRs are just one type of PRR, as there are others that are also extremely important for the normal function of immune and non-immune cells alike, including nucleotide-oligomerization domain leucine-rich repeat (NOD-

LRR) proteins and cytoplasmic caspase activation and recruiting domain helicases such as retinoic-acid-inducible gene I (RIG-I)-like helicases (RLHs) (Cinel, 2009).

The effects of the recognition of PAMPs or DAMPs by PRRs can vary, but in immune cells like macrophages, PRR ligands can activate a multimeric protein complex called an inflammasome. This consists of an inflammasome sensor molecule (such as a NOD-LRR), an adaptor protein called ASC (apoptosis-associated speck-like protein containing a CARD (Caspase recruitment domain)), and caspase-1 (Martinon, 2002). This inflammasome complex can be highly regulated. The best described inflammasome complex named NLRP3 (NOD-LRR and pyrin domain-containing 3) after its PRR protein that acts as a sensor for activation, is thought to be regulated by multiple signals. One of the signals thought to prime the activation of the inflammasome complex and the expression of pro-interleukin-1 β (pro-IL-1 β) is the binding of surface PRRs, including TLR2 and TLR4 (Bauernfeind, 2009). Other receptors can also be involved in the regulation of inflammasome complexes. For example, dectin-1, a receptor in the lectin family that can recognize fungal derived β -glucans, may play a role in priming cellular defenses including activation of the NLRP3 inflammasome complex through Syk kinase signaling (Rogers, 2005; Gross, 2005).

The end result of inflammasome activation is the cleaving of inactive pro-IL-1 β into active IL-1 β (Hernandez, 2011). IL-1 β is a pro-inflammatory cytokine produced by immune cells, like the macrophages described earlier that include the microglia of the central nervous system. IL-1 β has a variety of activities, but most importantly it is one of the main chemical mediators of inflammation in damaged or infected tissues (Garlanda, 2013). IL-1 β exerts its activity on cells by binding to its specific receptor IL-1R1, which is in the same superfamily as

the TLRs (Boraschi, 2013). IL-1 β has an effect often specific to the cell it binds to in many areas of the body, but inflammation is a common result.

Inflammation is a bodily response triggered by infection or tissue injury that involves the delivery of blood components to the site of the noxious stimuli. As described, it is often triggered initially by the binding of the appropriate ligands to surface or intracellular PRRs. Activated inflammasome complexes then produce proinflammatory cytokines that act as the primary chemical mediators, resulting in inflammation. Activated endothelium allows selective permeability to plasma and leukocytes into the tissue, causing local swelling. Pain plays an important role in inflammation, as it alerts the host organism to the abnormal state of the damaged tissue (Medzhitov, 2008).

Zymosan is a preparation of *Saccharomyces cerevisiae* cell wall components that can be used as a potent inflammatory stimulus *in vivo* and *in vitro*. It is composed mostly of β -glucans, mannans, mannoproteins, and chitin. All of these components can potentially activate an innate immune response by allowing myeloid cells to recognize a fungal organism (Gantner, 2003). TLR2 and dectin-1 both recognize the constituents of zymosan and are the primary PRRs for triggering the immune response. Dectin-1 is primarily responsible for triggering the phagocytosis of zymosan particles so that macrophages and other phagocytic myeloid cells such as dendritic cells or neutrophils can internalize and process it (Brown, 2001). Internalization of these particles further the efficiency of the inflammasome complexes inside the cells that produce active IL-1 β , leading to an inflammatory response.

The nervous system can be divided into two different entities, the central nervous system (CNS) and the peripheral nervous system (PNS). The afferent branch of the PNS carries sensory information from sensory receptors in the skin, mucous membranes, and internal organs to the

spinal cord. Mostly localized to the dorsal horn of the spinal cord, this sensory information travels to the brain for processing and an efferent response is often sent back to the original location of the sensory stimulus. Primary sensory neurons span from their receptive fields to the dorsal horn of the spinal cord (Hogan, 2010). The cell bodies of these neurons reside in ganglia located just lateral to the spinal cord on both sides in a small cavity inferior to the pedicle of the vertebrae.

These sensory ganglia termed dorsal root ganglia (DRG) contain only the cell bodies of primary afferent sensory neurons that transmit information to the CNS, some of which are nociceptive and are involved in the transmission of pain signals. (Hanani, 2005). DRG neurons in the sensory ganglia are surrounded by satellite glial cells, which may play a role in modulating pain transmission to the CNS (Costa, 2015). DRG neurons have shown plasticity in their signaling and expression. Nociceptive neurons show an ability to hypersensitize in response to large inputs of signaling generated by particularly noxious stimuli by a variety of different methods (Ji, 2001).

SP is released from primary afferent neurons to convey information regarding the presence of noxious stimuli. SP represents the excitatory transmitter released in the transmission of pain, so higher levels of expression likely represents increased perception of pain (Jessnell, 1979). The sensitivity and expression of SP can be modulated by other cells. It has been shown that activated glial cells can secrete chemical mediators that modulate SP release, including IL-1 β secreted in the dorsal horn of the spinal cord resulting in hyperalgesia (Watkins, 2001; Sweitzer, 1999).

This modulation of pain and SP expression in primary sensory neurons by interaction with IL-1 β has been a concept under investigation for quite some time. IL-1 β has been shown to

interact with nociceptive neurons in a variety of ways that lead to increased perception of pain (Samad, 2001; Kawasaki, 2008; Üçeyler, 2009). The direct induction of SP release by IL-1 β has also been shown in primary cultures of rat DRG cells (Inoue, 1999). The interactions between pro-inflammatory cytokines and nociceptive sensory neurons in the DRG are not completely understood, but much interaction has already been described.

DRG neurons contain PRRs similar to the ones seen in myeloid cells, and both neuronal and non-neuronal cells in the CNS express inflammasome protein complexes seemingly identical to those seen in macrophages and other cytokine secreting cells (Santoni, 2015). Increasing evidence has demonstrated that TLRs expressed in primary sensory neurons are involved in pain and itch sensation (Liu, 2012). In chemosensitive carotid body glomus cells, tissue of neural crest origin like DRG neurons, it has been shown that binding of TLR-2 by zymosan particles upregulated the expression of NLRP3 inflammasome proteins, resulting in expression of IL-1 β (Ackland, 2013). This IL-1 β resulted in increased sensitivity of the chemosensory cells, thus acting in an autocrine manner to enhance sensory signaling. It is also known that cytokines produced in situations of peripheral inflammation interact with IL-1 β receptors on vagal afferent nerves and glomus cells constituting a direct instance of communication between the innate immune system and nervous system (Matteoli, 2012).

In this experiment, zymosan is used to induce inflammation in the peritoneal cavity of mice. We hypothesize that macrophages recruited to the site of inflammation will produce pro-inflammatory cytokines, namely IL-1 β , which will cause an upregulation of expression of SP in DRG neurons and in their sensory nerve processes of the dorsal horn in the spinal cord. This upregulation could be the direct effect of communication between the innate immune system and

primary sensory neurons or a result of those neurons detecting the presence of the PAMPs in zymosan directly.

Materials and Methods:

Animals:

Adult male C57BL/6 mice purchased from Harlan Laboratories were used for this study; animal subjects ranged from two to four months in age. Animal protocols were approved by the East Tennessee State University Committee on Animal Care and conformed to guidelines of the National Institutes of Health as published in the *Guide for the Care and Use of Laboratory Animals* (Eighth Edition, National Academy of Sciences, 2011).

Zymosan model of systemic inflammation:

Experimental adult male mice were treated with an intraperitoneal (i.p.) injection of a 200 μ l zymosan (Sigma Z4250) suspension in sterile-filtered normal saline using a 22 gauge 1 inch needle. The dose of zymosan in the suspension was 500 mg/kg based on the weight of each individual mouse. The zymosan suspension was prepared in the following manner. Zymosan was measured and sterilized using UV light for 30 minutes. Sterile-filtered saline was added to the zymosan and the suspension was vortexed extensively, kept at room temperature overnight, and vortexed again just before use to ensure thorough mixing. Control mice were treated with an i.p. injection of 200 μ l of sterile-filtered saline. Sterile-filtered saline (1 mL) was administered

subcutaneously (s.c.) to both groups of mice at the time of injection in order to maintain adequate fluid volume.

Tissue Collection:

The rectal temperature of each mouse was recorded at 24 hours after treatment, and mice were euthanized with isoflurane. After confirmed death, the thoracic cavity of the mouse was opened rapidly, the wall of the right atrium was cut, and a blunted 22 gauge needle was inserted into the left ventricular chamber of the heart for perfusion of the vascular system. Using a peristaltic pump (Masterflex® DigiStaltic®), the vascular system was flushed at a rate of 10mL per minute with 40 mL of 0.1 M phosphate buffered saline (PBS) in order to clear all blood from the circulation and tissues. To prevent clotting, 1 unit per mL of heparin sodium (Sagent™ NDC #25021-400-30) was added to the PBS solution. The same method was used to perfuse the mice with 40 mL of ice cold 4% paraformaldehyde (PFA) in PBS to fix all tissues.

After fixation, all skin and connective tissue was removed from the dorsal side of the animal. Two long vertical cuts were made immediately lateral to the vertebral column spanning from the sacral region to the cervical region. All muscle and connective tissue on top of the lower thoracic and lumbar region of the vertebral column was removed, and a rongeur was used to remove the entire dorsal half of the vertebra. This was done by squeezing the transverse processes, breaking the pedicles, and removing the remaining bone, exposing the spinal cord (SC) laying underneath in the vertebral foramen. The SC section directly medial to the last rib is T13, and directly posterior to that is L1 (Malin, 2007). All DRG and SC sections in this experiment are at the L1 level. The fixed SC was carefully cut with a razor blade, severed from

the dorsal root, and removed. It was then transferred to a microcentrifuge tube containing cold 4% PFA in PBS. The DRG sitting on both sides were carefully located, the attached nerve tissue going into the periphery was cut, and the L1 DRG were removed and immediately placed into cold 4% PFA solution as well. This entire process was done for every mouse in both experimental and control groups. All tissues were post-fixed overnight at 4°C.

Tissue Preparation:

All tissue was transferred to 20% sucrose in PBS after remaining in 4% PFA overnight. Once the tissue sank to the very bottom of the microcentrifuge tube (indicating that it is saturated with the sucrose solution), it was carefully removed and frozen on powdered dry ice. Due to the small size of the DRG, special preparation was necessary. One fresh liver was removed from a euthanized mouse. A small piece was taken from the liver, and a cut was made to form a small depression in the rounded anterior face of the piece of liver. A single DRG was then placed in this depression, and the entire piece was frozen by placing it on the dry ice. This allowed the DRG to be more easily mounted onto the specimen plate to eventually be cut on the cryostat. SC sections were placed directly onto the dry ice to freeze instantly. Once the tissue was frozen, it was affixed to a specimen plate using mounting media (Sakura® Tissue-Tek O.C.T. Compound 4583). This tissue was then cut in a cryostat (Leica CM3050 S) at -20°C. The DRG sections were cut at 20 µm and the SC sections were cut at 30 µm and affixed to charged slides. Slides with dried tissue attached were stored at -80°C and not thawed until right before the immunofluorescence staining process was started.

Immunostaining:

Slides were removed from the -80°C freezer and slowly allowed to thaw to room temperature. The slides were then placed in coplin jars and thoroughly washed with 0.1 M PBS. Next, the slides were washed with a detergent solution containing bovine serum albumin (0.5% BSA + 0.4% Triton-X100 +0.1M PBS (pH 7.3)). The slides were then placed into an incubation box and covered in a blocking buffer (5.0% normal donkey serum + 1.0% BSA + 0.4% Triton-X100 +0.1M PBS (pH 7.3)). The slides remained in blocking buffer for two hours at room temperature. After the two hours, the blocking buffer was replaced by the primary antibody solution (1:500 Rabbit anti-SP (Immunostar #20064) antibody + blocking buffer used above) and allowed to incubate overnight at room temperature. DRG sections were double labeled with the primary antibody solution also containing 1:250 Guinea Pig anti-PGP9.5 (Neuromics GP14104) which acted as a general marker staining all neuronal cell bodies and axons. SC sections were not double labeled, and were only subjected to the SP primary antibody.

The following day, the slides were rinsed again with first PBS and then the detergent solution. The tissue was then covered in blocking buffer once again for an hour at room temperature, and then replaced with the secondary antibody solution (1:200 Donkey anti-Rabbit IgG Alexa Fluor® 488 (A-21206) + blocking buffer) for two hours. The secondary antibody applied to the DRG sections also included 1:200 Goat anti-Guinea Pig IgG Alexa Fluor® 594 (A-11076). All SP expressing neurons in the section fluoresced green, and all neurons fluoresced red when observed under the correct correlating microscope filter. SC sections do not receive the anti-Guinea Pig secondary antibody since they did not receive the PGP 9.5 primary antibody, so only green fluorescence was present.

The slides are then rinsed with 0.1 M PBS, mounting media (Citifluor AF1 No. 19470) is applied, and cover slips are placed and sealed. This entire immunofluorescence staining protocol is standard in Dr. Hoover's lab (Department of Biomedical Sciences, ETSU) and the expression of the desired protein in the tissue can be seen with fluorescence microscopy. Substance P was stained for in this manner and compared between the control mice and the zymosan suspension treated mice. Green and red fluorescence images of DRG sections were captured using confocal microscopy (Leica TCS SP8 Confocal Microscope), while images of green fluorescence in SC sections were captured using with the Olympus® Q-Color 3 digital microscope camera. Both types of sections were captured using a 10x objective lens. For the confocal images of DRG sections, maximum projection images were used. This allowed many images to be acquired in a series of different focal planes for each section, and then combined into a single high definition image of a single focal plane. SP expressing neurons (green) and total neurons (red) in DRG sections were counted using Stereo Investigator (MBF Bioscience) software and the images were merged in order to obtain the sample images included in this report.

For SC sections, positive SP nerve fiber density was calculated as percent of total area using ImageJ (U. S. National Institutes of Health) software. The total area used in the calculations of these values was determined as consistently as possible by following a standard protocol for determining the region of interest. This region of interested included approximately one eighth of the entire spinal cord section so that a single dorsal horn region could be analyzed. This analysis process was checked and protected from experimental bias by a process involving two individuals, where one randomly assigned names to the image files and kept track of the true identity of each, and the other analyzed the image using the procedure mentioned. All statistical

data and graphs were generated using GraphPad Prism 6 software (GraphPad Prism version 6.00).

A complete descriptive statistics analysis was done and a p-value was obtained using an unpaired t test. There was n=3 for both saline control mice and the zymosan treated mice for the DRG experiment. For the SC analysis, there was an n=5 for both groups of mice. This difference between experiments was due to the difficulty of successfully collecting DRG compared to the relatively easy process of collecting SC sections. Only one DRG section per animal was include in the final analysis in the DRG experiment. For the SC experiment, three dorsal horn sections per animal were included in the final analysis. A p-value of less than 0.05 was the criterion for judging significant differences.

Methods Discussion:

The methods for tissue collection, preparation, and immunostaining changed extensively over the course of this project. Obtaining images of a high enough quality and consistency to reliably analyze proved difficult so a trial and error approach was used. Initially, the perfusion of the deceased mouse was performed with 30 mL of normal saline solution using a syringe and a 22 gauge needle. The fluid was injected directly into the left ventricle, and the same was done with a separate syringe with 30 mL of 4% PFA for the fixation of the tissue. The extraction was done the same way as described, however the success rate of locating, carefully extracting, and preserving the DRG was very low until substantial experience was gained. The extracted tissue was placed in a microcentrifuge tube containing 10% buffered formalin phosphate and remained in this solution for two consecutive days.

The tissue was then processed, dehydrated, and embedded into paraffin blocks. Sections 5 μm thick were cut using a microtome (Microm HM 310) and affixed to slides. Before immunostaining could continue, these slides had to go through a rehydration and antigen retrieval process. Sections going through this protocol did not stain consistently or specifically enough to use in the experiment. This may have been an issue of over fixing the tissue, or a failure of the antigen retrieval process possibly due to SP being such a small protein or the fragile nature of nervous tissue.

Results:

Experimental Data and Statistics

Value	Saline Control	Zymosan Treated (500mg/kg)
Mean \pm SEM (DRG) Neurons expressing SP as percent of Total Neurons	14.87% \pm 0.4256%, n=3	17.77% \pm 0.4807%, n=3
Mean \pm SEM (SC) Area of SP expressing nerve fibers as percentage of total area	21.63% \pm 0.7458%, n=5	25.43% \pm 0.9842%, n=5
DRG Experiment p-value	0.0107*	
SC Experiment p-value	0.0153*	

Table 1: Statistical Data. This table lists the key statistical results of the experiment. The p-value was determined using a two-tailed unpaired t test. Both p-values indicate that the results for each experiment are statistically significant.

Table 1 shows the statistical results of both the DRG and SC experiments calculated using the GraphPad Prism software. The percent of total neurons expressing SP was increased as a result of the zymosan treatment. The DRG sections from the zymosan treated mice showed a

19.5% increase of neurons expressing SP as a percentage of total DRG neurons counted. For the SC experiment, a 17.5% increase in total nerve fiber density as a percentage of total area due to the zymosan treatment was calculated.

Figure 1 is a graphical representation of the data from the DRG experiment in which a clear increase in SP expressing neurons can be seen. Sample images created during the experiment are included with the figure. These images are merged examples of both the red and green fluorescence images in which the percentage of total neurons expressing SP can be most easily seen. When the analysis was performed, the merged images were not used. Figure 2 is a graphical representation of the results for the SC experiment which also shows a clear increase in SP expressing nerve fibers. The sample images in the figure are two of the actual images used for the analysis, and an increase in green fluorescence can easily be seen.

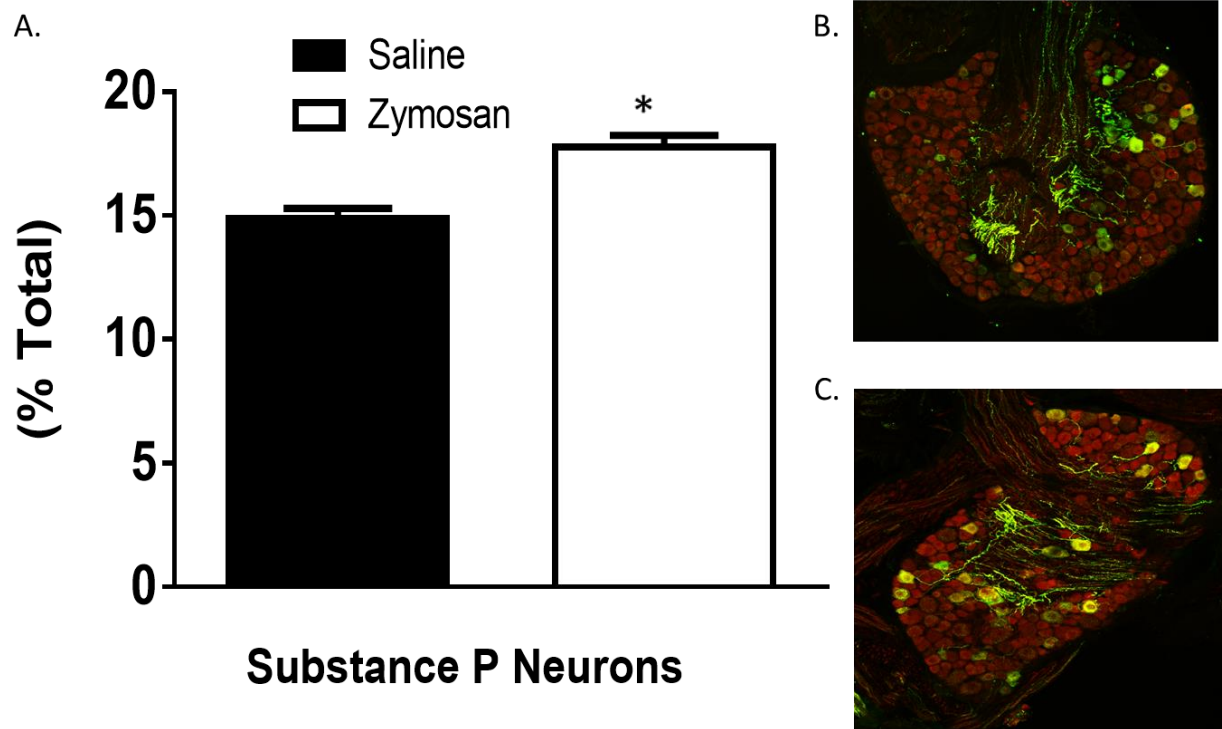


Figure 1: The effect of zymosan-induced peritonitis on substance P expression of dorsal root ganglion neurons. A. This graph shows the mean percentage of total neurons that show positive immunofluorescence staining for the expression of substance P for both the saline (control) injected mice and the zymosan (500mg/kg) injected mice. B. A representative image of a DRG section from a saline injected mouse. (Red color /PGP 9.5; Green/SP) C. A representative image of a DRG section from a zymosan injected mouse. (Red color/PGP 9.5; Green/SP)

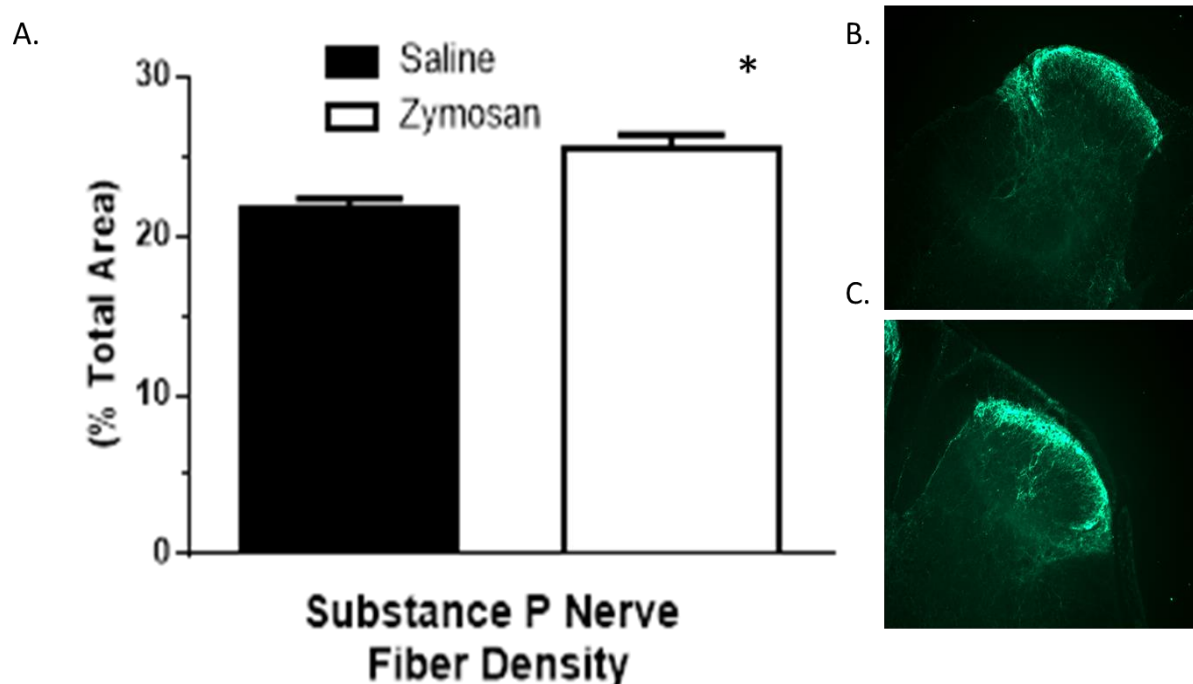


Figure 2: The effect of zymosan-induced peritonitis on substance P expression of spinal cord nerve fibers around the dorsal horn. A. This graph shows the mean percentage of total area that show positive immunofluorescence staining for the expression of substance P for both the saline (control) injected mice and the zymosan (500mg/kg) injected mice. B. A representative image of a SC section from a saline injected mouse. (Green/SP) C. A representative image of a SC section from a zymosan injected mouse. (Green/SP)

Discussion:

The results of this experiment show a 19.5% increase in the ratio of SP expressing neurons as a function of total neurons due to the zymosan treatment as compared to the saline control. We hypothesized that this increase would happen according to the current literature on the topic, but the consistency of this result was better than expected. The sections of tissue examined are only small slices of the DRG as a whole, but the consistency of the increase seen from all the sections suggests that this is probably happening throughout the entire DRG. It is not clear according to this experiment whether this is happening at all spinal levels, as only DRG from the L1 vertebral level were examined in order to produce a consistent comparison of data.

L1 is a spinal level that is expected to have many projections into the peritoneal cavity, useful for this experiment. This may make it more likely to be affected by the different potential causes of this increase in SP expression.

It is not entirely clear what the mechanism behind this increase is, but it could be a number or combination of different things. Most likely, inflammatory cytokines, produced by the macrophages recruited to the site of injection, are binding to IL-1R1 receptors on the DRG processes in the cavity or on the cell bodies. The current literature has shown that IL-1 β does increase the expression of SP and can even directly induce its release, so this explanation may be the main driving force. It is also possible that TLR2 receptors on the DRG processes in the peritoneal cavity are reacting directly to the zymosan compound, but this is not as convincing as the TLR2 and Dectin-1 receptors on the macrophages are much more numerous and more likely to cause a significant change like we are seeing. It is interesting how quickly the zymosan treatment had an effect on the SP expression in the two tissues tested, as the mice were euthanized only 24 hours after the treatment. Further work could be done to see what chronic effects could take place after this treatment in survivors. Different doses of the zymosan compound would likely need to be considered in this case due to the lethality of the (500mg/kg) dose used in this experiment.

It is also not known what the source of the new expression of SP is. Neurons expressing low levels of SP could be getting hyper sensitized and expressing much higher levels of SP in response to the inflammatory cytokines, or the neurons of the DRG could be undergoing remodeling. Neuropeptide expression by cells has been seen to change in response to peripheral nerve injury, and a similar mechanism may be involved in this response to a violent inflammatory response to zymosan (Villar, 1989). More in depth experimentation and review of

the literature may be able to shed light on this process. It would be beneficial to see if and how satellite glial cell activation changes as a result of the zymosan induced peritonitis. This could be done by staining for glial fibrillary acidic protein (GFAP), a protein expressed when glial cells are activated. It is known that activated glial cells in the DRG can have a dramatic effect on the neurotransmission and peptide release of the nociceptive neurons (Costa, 2015) (Watkins 2001).

Interestingly enough, the increase in SP expression due to the zymosan treatment as compared to the saline control group seen in the SC sections was a similar value at 17.5%. It would be very surprising to see such a consistent increase of SP expression in the DRG section and not to see it in the SC at the same local levels. The nerve fibers we see in the spinal cord at the dorsal horn are the axons from the DRG neurons relaying their sensory information to the CNS. There are second order neurons in the SC that can receive and amplify the afferent signal, and it was noted in the introduction that microglial cells in the SC have been shown to produce IL-1 β and cause a central sensitization effect resulting in increased afferent signaling. IL-1 β secreted by zymosan activated macrophages may have the same effect if it reaches these neurons in the spinal cord.

Just as with the DRG, the mechanism of this increase in this experiment is not exactly known. There could be a couple of different explanations for this effect, including that this increase is just a direct effect of the increase of expression from the neurons in the DRG. If more DRG cell bodies are expressing SP, then more axons in the SC should be expressing it since they are one in the same cell. Another possibility is that there is a sprouting of nerve fibers that express SP. This increase in nerve fibers expressing SP could be especially important to understand when dealing with conditions of chronic pain and hyperalgesia. The increase in

nociceptive afferents and perceived pain in patients experiencing inflammation due to sepsis is an important issue that needs to be better understood.

There was a potential for bias in this experiment, as the size, brightness, and morphology of each section was not identical, so making judgment calls was necessary during the analysis of SC sections. This posed an issue when deciding the region of interest and the threshold levels in the analysis of the images. The attempt to avoid this possible issue by evaluating the results of the image in a blind manner seems to have negated a lot of the potential bias. Analyses were done multiple times and extremely similar values were obtained each time a section was repeated.

Understanding these results and the mechanisms responsible for the increase in SP expression could be extremely helpful in the ongoing battle with states of chronic hyperalgesia seen in sepsis survivors due to systemic inflammatory responses (Zimmer, 2006). It is likely that a combination of pathways are leading to this phenomenon, but with better understanding may come novel and more effective ways in treating this lasting symptom of a complex and dangerous condition.

Conclusion:

This experiment has shown that zymosan-induced peritonitis causes an upregulation of substance P expression in both dorsal root ganglia neuron cell bodies and nerve processes in the SC, especially localized in the dorsal horn where sensory information enters the central nervous system and ultimately reaches higher centers where pain perception occurs. This may represent a hyper sensitization of the peripheral nervous system to noxious stimuli resulting in increased perceived nociception. The specific mechanism causing this effect is not confirmed in this

experiment, however many possible pathways are explained and considered. Further experimentation using tools such as mice strains with gene knockouts could be useful in learning more about this process. For example, mice with Dectin-1 gene knockouts could help determine the importance of phagocytosing the individual beta glucan molecules from the zymosan treatment to producing the inflammatory response that leads to the upregulation of expression of SP in the DRG and SC.

The methods used in this experiment could prove to be invaluable in further studies. The method of locating, isolating, extracting, processing, and staining dorsal root ganglion sections from mice provides a means of learning more about these cells in conditions of induced inflammation or other relevant conditions. Many other proteins of interest in both dorsal root ganglia and spinal cord sections could be researched further in the same manner as substance P was investigated in this study. Valuable insight about zymosan-induced peritonitis could be learned by exploring the effects of this condition on the levels of interleukin 1 β , glial fibrillary acidic protein, c-fos, TLR2, and markers for nerve sprouting. Further understanding of the zymosan-induced inflammatory model could provide useful insight on its use in developing treatments or preventative measures in patients experiencing dangerous inflammatory responses.

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