Neuronal control of localized inflammation through expressed nicotinic acetylcholine receptors: a study carried out in mice

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Abstract

Introduction Although the local inflammatory reactions are known to be regulated through cholinergic anti-inflammatory pathways, the exact subtypes of nicotinic acetylcholine receptors involved in neuroimmune modulation are not well identified.

Objectives Immunohistochemical localisation of α1 subunit of nicotinic acetylcholine receptors (α1nAChR) in sites of localised inflammation induced by injecting turpentine to the hind limbs of Balb/C mice.

Methods Localised inflammation and subsequent development of sterile abscesses was induced by injecting sterile turpentine subcutaneously into thighs of Balb/C mice. Sterile saline was used in the control. Skin and muscle tissues of inflammatory sites were recovered from the animals after 48 hours and were stained with hematoxylin and eosin. Indirect immunohistochemistry was done using anti-α1nAChR as the primary antibody and biotinylated anti-rat IgG as the secondary antibody. Labeled streptavidin biotin (LSAB) technique was used with diaminobenzedene to detect the immunoreactivity (IR). Intensity of immunostaining was determined based upon a score of 0 - 3+ by qualitative computerised image analysis using FSX 100 Olympus microscope.

Results H and E stained slides showed polymorphonuclear leukocytes (PNL) infiltration at the abscess sites while the saline injected control tissue sections did not show PNL infiltration. A 2+ immunoreactivity (IR) of α1nAChRs was visible at peripheral zones of sterile abscesses where PNL infiltrations were high while the central area with necrotic tissue did not show IR. A subcutaneous lymph node found within the inflammatory region expressed IR of α1nAChR in its capsular sinuses, subcapsular sinuses and trabecular regions.

Conclusions The findings suggest the possible role of controlling localised inflammatory response by parasympathetic cholinergic nerves through α1nAChRs of inflammation sites.

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Introduction

The central nervous system is known to interact with the immune system through the autonomic nervous system and modify immune responses. Though the sympathetic system is the main component which controls the functions of lymphoid tissue, recent evidence shows significant influence by the cholinergic nervous system as well [1, 2]. Accordingly cholinergic anti-inflammatory mechanisms can inhibit the activation of macrophages and release of cytokines [2].

The cholinergic system, which uses acetylcholine as a neurotransmitter, is one of the excitatory pathways of the central nervous system [2]. Acetylcholine and multiple nicotinic acetylcholine receptor (nAChR) subunits are present on many cell types including endothelial cells and cells of the immune system [3, 4]. Thymocytes, thymic epithelial and myoid cells of immune organs express nAChRs on their cell surface [5]. Peripheral T cells express an unusual profile of muscle type nicotinic subunits, but their nAChR expression has not been identified [6]. In addition, expression of mRNA of α5, α9, α10, β1, β2, β4 nAChR subunits in the freshly isolated CD4 and CD8 T cells have been reported [7]. Gene expression of α1, α7 and α10 nAChRs also has been shown in mRNAs of primary human macrophages isolated from blood mononuclear cells [8].

Majority of the evidence shows the expression of α7nAChRs on monocytes and macrophages. [3]. α7nAChR is involved in the anti-inflammatory pathway [8]. Similarly, several studies show the crucial role of α7nAChR in the inhibition of excessive inflammation of many organs in the neuroimmune network [9, 10]. But the anatomical distribution of other nAChRs or their functional effects has not been studied.

A recent study shows that the parasympathetic cholinergic innervations are found predominantly in lymph nodes and spleen parenchyma in the supporting framework of reticulin fibres and consists mainly of α1nAChRs with minimum of α7nAChR in T cell and macrophage abundant regions of these tissues [11].
The α1 subunit of the nAChR is described as potential α-bungarotoxin-binding receptor in mammalian cells and experimental findings indicate the importance of these receptors in the inhibition of TNF synthesis in vitro by acetylcholine [8]. Studies are sparse regarding the distribution and functional role of α1 subunit of the nAChR at the site of inflammation.

The aim of our study was to investigate the distribution of α1 subunit of nAChRs in the sites of local inflammation induced by sterile turpentine injection [12]. In mice, it induces the formation of discrete aseptic abscesses without detectable injury to other tissues [12]. Findings of our study would provide the anatomical basis to investigate the role of α1nAChRs on the localised inflammatory response.

**Methods**

Balb/C mice (male, n=3) aged 7-8 weeks were purchased from Medical Research Institute and were housed individually in filter-top plastic cages and handled according to WHO guidelines for animal handling.

Sterile tissue damage (localised inflammation) was induced with commercial grade pyrogen free sterile turpentine filtered through 0.2 μm pore size sterile filters (Merck Millipore, Germany) to remove all microbes. Thirty micro liters (30μl) of pure undiluted turpentine oil (100%) was injected subcutaneously into both hind limbs of lightly anaesthetised (inhaled dimethyl ether) Balb/C mice (n=2). Injection was done with a 1 ml insulin syringe fitted with a 29½ gauge needle. The experiment was performed in two animals. Pyrogen free sterile saline was used as the control injection in one Balb/C mouse.

The abscess formation was monitored in 12 hour intervals in two groups. After 48 hours, some visible abscesses were localised in the dorsal surface of the hind limbs of the turpentine injected animals. The mice were killed after 48 hours.

The skin and muscle tissues were carefully recovered from the inflammatory site and were immediately fixed in 10% formaline saline. The tissues were processed for preparation of hematoxylin and eosin (H and E) sections. The slides were observed under light microscope for histological changes in the site of control animals [Plate 1(a)]. The micro-structural features were similar to healthy tissue.

Histopathology of sterile abscesses

On macroscopic examination (after 48 hours) swelling was visible and the abscesses had formed in the sterile turpentine injected sites of the hind limb of the Balb/C mice. In the control Balb/C mice there were no visible changes in the sites of sterile saline injection. The sterile saline injection did not cause any significant histological changes in the site of control animals [Plate 1(b)]. The micro-structural features were similar to healthy tissue.

The sterile turpentine injection caused a localised inflammation in the skin, subcutaneous tissue and deep skeletal tissues. The H and E stained abscess tissue sections with localised inflammation exhibited a large accumulation of PNL near the site of turpentine injection in the hind leg of Balb/C mice [Plate 1(b)]. The centre of the abscess had large necrotic tissue elements such as fibrin, keratin squames, degenerating PNL, macrophages and serous fluid and it was rimmed by surrounding zone of active PNL, lymphocytes and macrophages. The PNL,
Plate 1. Abscess tissue (H & E stain)
(a) Dermis of the skin and the subcutaneous tissue of control Balb/C mice, bar = 45μm
(b) Subcutaneous tissue of the thigh of Balb/C showing the abscess formation, bar = 45μm

Plate 2. Abscess and subcutaneous lymph node, IHC of α1nAChR
(a) Skeletal muscle of Balb/C tongue, α1nAChR positive, S- striations, N-nucleus, bar = 15 μm
(b) Lingual salivary gland of Balb/C mice, α1nAChR negative, G- glandular tissue, bar = 10 μm
(c) Abscess tissue and related tissues, bar = 102 μm
(d) Subcutaneous lymph node showing α1nAChR positive IR, bar = 20 μm
lymphocytes and macrophages were scattered in between the skeletal muscle tissue and prominent lymph nodes were found in the vicinity of the injected sites.

**Immunohistochemistry of sterile abscesses with α1nAChR antibody**

The positive control showed skeletal muscle tissues of the Balb/C mice treated with α1nAChR antibody expressing a strong diffuse dark brown colour with absence of α1nAChR antibody immunoreactivity [Plate 2(a) and 2(b)]. A 2+IR was visible at peripheral zones of sterile abscesses where PNL infiltrations were high for α1nAChRs while the central area showed less IR [Plate 2(c)]. The subcutaneous tissue found in the vicinity of the abscess showed scattered IR of α1nAChR antibody. A subcutaneous lymph node associated with the inflammatory region expressed α1nAChR IR in its capsular, subcapsular sinuses and trabeculae regions [Plate 2(d)]. Skeletal muscle tissue found in the inflammatory area expressed 2+IR, while the leukocytes found in between the muscle tissue expressed less IR of α1nAChRs.

**Discussion**

Sterile turpentine is a known inflammatory agent which stimulates inflammation [16]. Accordingly, injection of sterile turpentine induced localised inflammation in the subcutaneous region of hind thigh of mice. The infiltration of neutrophils and macrophages and the histopathological changes of the inflammatory site in the present experimental study were similar to the reported histopathological changes of local inflammation produced by infectious agents [17]. PNL infiltration was significantly higher in test tissues, while in the control tissue there was no PNL distribution.

Sterile turpentine stimulates inflammation by increasing the level of certain serum glycoproteins and the acute phase reactants in circulation and induces the local synthesis of interleukin-1β and TNFα [12,16]. Similarly a marked elevation of levels of the major murine acute-phase proteins, serum amyloid A and serum amyloid P in plasma has been reported after 48 hours of turpentine injection [16]. The previous documented studies promoted us to apply sterile turpentine as the irritant to induce sterile inflammation.

The crucial role of cholinergic anti-inflammatory pathway is exerted by potential inhibitory control of inflammation [9,10, 19]. Neural regulation of acute localised inflammation is by the cholinergic and anti-inflammatory pathway known as the “inflammatory reflex”. Inflammatory stimuli activate afferent pathways within autonomic nerves like the baro reflex. Relaying in the hypothalamus it elicits an efferent anti-inflammatory response that rapidly prevents the overproduction of inflammatory cytokines. The nervous system integrates the inflammatory response [9]. The autonomic nervous system detects the inflammatory stimuli such as local irritants and produces cytokines. Afferent signals are also thought to be transmitted by autonomic nerves and activate the efferent autonomic nerve signalling and release of ACh in the vicinity of macrophages. In the cholinergic anti-inflammatory pathway, the efferent arm of the inflammatory reflex is composed of the efferent vagus nerve, the neurotransmitter ACh and the α7 subunit of nAChR [20]. Based on this concept attempts can be made to treat autoimmune diseases.

In this process acetylcholine can interact with α7nAChRs expressed by macrophages and other cytokine-producing cells, down-regulate proinflammatory cytokine synthesis, and prevent tissue damage [8]. In this study we have shown the expression of α1nAChRs in sites of local inflammation, suggesting the possible role of these nAChRs in the proposed cholinergic anti-inflammatory pathway. Similar to α7 subtype α1nAChRs expressed in the inflammatory site may inhibit inflammation by suppressing cytokine synthesis.

α1nAChRs are known to be α-Bungarotoxin binding receptors. These α-Bungarotoxin affinity nAChRs are known to produce cross bridges between cholinergic nervous system and immune system. Therefore the findings of the present study would be beneficial in understanding the cholinergic anti-inflammatory pathway.

Although, α1nAChRs were identified in the areas of PNL infiltration, even with higher magnification it was difficult to identify the exact cell type expressing the IR. As the abscess site attracted a large amount of macro-phages and PNL, we could assume that α1nAChRs are expressed in the live cells of the peripheral rim of the abscess. This suggests that α1 subtype of nicotinic receptors may play a role in the anti-inflammatory response in localised inflammation. The subcutaneous lymph node associated with the inflammatory region also expressed α1nAChR IR in its capsule, subcapsular sinuses and trabeculae regions. This is also suggestive of anticho-linergic control of inflammation.

This study was limited to α1nAChR due to the high cost of the primary antibody. Further investigations of the expression and distribution of cholinergic nerves by using the primary antibody markers such as the anti-vesicular acetylcholine transporter protein antibody and anti-choline acetyl transferase antibody would be useful. The regional lymph nodes draining these local lymph nodes can investigated for the expression of α1nAChRs.

In conclusion, the findings confirm the higher expression of α1nAChRs at the inflammatory sites. This is suggestive of neuronal control of localised inflammatory response by parasympathetic cholinergic innervation through α1nAChRs.
Conflicts of interests
There are no conflicts of interest.

References