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• BASIC RESEARCH •

Evidences for vagus nerve in maintenance of immune balance and transmission of immune information from gut to brain in STM-infected rats

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Abstract

AIM: **To determine whether Salmonella Typhimurium (STM) in gastrointestinal tract can induce the functional activation of brain, whether the vagus nerve involves in signaling immune information from gastrointestinal tract to brain and how it influences the immune function under natural infection condition.**

METHODS: **Animal model of gastrointestinal tract infection in the rat was established by an intubation of Salmonella Typhimurium (STM) into stomach to mimic the condition of natural bacteria infection. Subdiagphragmatic vagotomy was performed in some of the animals 28 days before infection. The changes of Fos expression visualized with immunohistochemistry technique in hypothalamic paraventricular nucleus (PVN) and superaoptic nucleus (SON) were counted. Meanwhile, the percentage and the Mean Intensities of Fluorescent (MIFs) of CD4+ and CD8+ T cells in peripheral blood were measured by using flow cytometry (FCM), and the pathological changes in ileum and mesenteric lymph node were observed in HE stained sections.**

RESULTS: **In bacteria-stimulated groups, inflammatory pathological changes were seen in ileum and mesenteric lymph node. The percentages of CD4+ T cells in peripheral blood were decreased from 42%±4.5% to 34%±4.9% (P<0.05) and MIFs of CD8+ T cells were also decreased from 2.9±0.39 to 2.1±0.36 (P<0.05) with STM stimulation. All of them proved that our STM-infection model was reliable. Fos immunoreactive (Fos-ir) cells in PVN and SON increased significantly with STM stimulation, from 189±41 to 467±62 (P<0.05) and from 64±21 to 282±47 (P<0.05) individually, which suggested that STM in gastrointestinal tract induced the functional activation of brain. Subdiagphragmatic vagotomy attenuated Fos expression in PVN and SON induced by STM, from 467±62 to 226±45 (P<0.05) and from 282±47 to 71±19 (P<0.05) individually, and restored the decreased percentages of CD4+ T cells induced by STM from 34%±4.9% to original level 44%±6.0% (P<0.05). In addition, subdiagphragmatic vagotomy itself also decreased the percentages of CD8+ T cells (from 28%±3.0% to 21%±5.9%, P<0.05) and MIFs of CD4+ (from 6.6±0.6 to 4.9±1.0, P<0.05) and CD8+ T cells (from 2.9±0.39 to1.4±0.34, P<0.05). Both of them manifested the important role of vagus nerve in transmitting immune information from gut to brain and maintaining the immune balance of the organism.**

CONCLUSION: **Vagus nerve does involve in transmitting abdominal immune information into the brain in STM infection condition and play an important role in maintenance of the immune balance of the organism.**

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INTRODUCTION

It has been suggested in recent studies that the vagus nerve, the tenth cranial nerve, might play an important role in transmitting immune information into the brain[1-5]. However, this conclusion is based on the experiments in which cytokines, endotoxins or exotoxins were usually used as immune stimulators through intraperitoneal or intravenous injection. All these immune stimulations, however, are non-natural and the role of vagus in natural infection condition has not been established yet. Salmonella Typhimurium (STM) belongs to the group B of Salmonella. It can infect both human beings and animals through gastrointestinal tract and leads to a local or general infection by inhibiting the host immune system[6]. Thus, in the current experiments we introduced Salmonella Typhimurium (STM) into stomach to mimic the natural bacteria infection in gastrointestinal tract and to reassess the role of vagus in transmission of immune signal by subdiagphragmatic vagotomy. The production of c-fos, an immediately early gene, has been used as a morphological marker of functionally activated brain neurons[7-15]. In the present study we observed the STM-induced Fos expression in hypothalamic paraventricular nucleus (PVN) and superaoptic nucleus (SON) and the effect of vagotomy. We also studied the importance of integrity of vagus nerve in the balance of T cell subpopulations.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley albino rats (180-210g, offered by Animal Center, Fourth Medical University) were used. Rats were housed individually in a temperature-controlled room in a natural light/dark cycle, with food and water available freely. The animals were trained for adaptation to handling and gastric intubation before the following procedures started.

Procedures

Subdiagphragmatic vagotomy Rats were anesthetized with pentobarbital sodium (40mg/kg, i.p.) and subjected to a complete subdiagphragmatic vagotomy (n=10) or sham operation (n=10). Briefly, after laparotomy, the two trunks of vagus were identified under an operating microscope. Both trunks were cut off close to the

diaphragm. For sham vagotomy, the vagus was similarly exposed but was not cut. After surgery, a recovery period of 28 days was allowed.

Preparation of STM Wild strain of STM (offered by Laboratory of Bacteria, Xijing Hospital, Fourth Medical University) was preserved in freeze-dried powder before use. In order to enhance the pathogenicity of the bacteria, STM were sub-cultured in mice abdomen (Kunming mice offered by Animal Center, Fourth Medical University) for 3 times and then the number of the bacteria was adjusted to 10^{10}/ml for use.

Intubation of STM Rats (n=20) were divided into 4 groups randomly, 5 for each. Group 1, saline(NS) + sham operation; 2, NS + vagotomy; 3, STM + sham operation; 4, STM + vagotomy. Food was taken away from the rats 24h prior to intubating STM or saline. After anesthetized with ether, all rats were intubated with 30g/L NaHCO3 300μl to neutralize gastric acid. Then the aminals of groups 3 and 4 were gastrically intubated with STM (10^{10} in saline, 1ml) and in the others (Groups 1 and 2) 1 ml of saline were given.

Perfusion and Sectioning After intubation for 22h, all rats were deeply anesthetized with pentobarbital (80mg/kg) and 1ml of blood was taken via heart as quickly as possible. The rats were then perfused transcardially with saline 100ml followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB) 500ml, pH 7.4, at 4℃. Blood was anti-coagulated with heparin. Brains, part of ileum and mesenteric lymph node were taken out and cryoprotected in 20% sucrose in 0.1 M PB overnight at 4℃. Frontal sections in 50μm-thickness were cut through whole brains with a microtome and collected in cold cryoprotectant and stored at -20℃ until immunohistochemistry processing. Serial ileum and mesenteric lymph node sections in 5μm-thickness were cut with a cyostat and mounted onto slides coated with gelatin and stored at -20℃ until histochemistry processing.

HE staining of ileum and mesenteric lymph node sections Slides of ileum and mesenteric lymph node were immersed successively in dimethylbenzene (10min×2), graded ethanol (100% 5min×2, 95% 2min, 80% 2min, 70% 2min and distilled water 2min), Harris hematoxylin (5-10min) and 10% acid ethanol for several seconds. After rinsed with tap water for 30min, slides were immersed again successively in distilled water (10-30min), graded ethanol (70%, 80%, and 95% 2min for each), 0.5% eosin (5-10min), 95% ethanol from several seconds to minutes, 100% ethanol (5min×2) and dimethylbenzene (10min×2). At last, the slides were sealed with gum and observed under a light microscope (Olympus B×60).

Flow cytometry (FCM) of blood T Cell Blood CD4+ and CD8+ Tlymphocytes were labeled by using indirect immunofluorecent labeling method. First, 80μl of anti-coagulated blood was incubated with mice anti rat CD4 mAb (1:100, Serotec company) or 15μl of mice anti rat CD8 mAb (1:100, Serotec company) for 30min at 4℃, then with 40μl of goat anti mice IgG-FITC (1:100, Serotec company) after washing twice with 0.01Mol/L Phosphate-buffered saline (PBS). FCM was used to detect the percentages and the Mean Intensities of Fluorescence (MIFs) of CD4+ and CD8+ T cells.

Immunohistochemistry of Brain Sections ABC immunohisto chemical technique was used to detecte Fos-immunoreactive (Fos-ir) cells in brain. One-in-five of brain sections were incubated with primary antibody raised from rabbit against Fos protein (Sigma Inc.) at a dilution of 1:3000. After incubation at room temperature for 36h, sections were rinsed with 0.01Mol/L Phosphate-buffered saline (PBS) (10min×3) and then incubated with biotinylated secondary antibody against rabbit IgG (Sigma Inc, diluted at 1:500) at room temperature for 4h. After rinsing with 0.01Mol/L PBS (10min×3), sections were incubated with avidin-biotin-horseradish peroxidase (1:500, Sigma Inc.) at room temperature for 2h. The reaction product was visualized with amine nickel sulfate-enhanced 3,3'-diaminobenzidine (DAB) method. The sections were dehytrated in graded ethanol, cleared with dimethylbenzene, and coverslipped with gum.

Counting of Fos-ir cells Sections of hypothalamus were observed with a light microscope (Olympus BX60). The number of Fos-ir cells was quantified by counting immunostained nuclei in PVN or SON at two consecutive typical sections with an image analysis system (Leica Quantimet 570 C). The number of Fos-positive nuclei in PVN or SON was the group mean ± SE.

Statistical analyses All data were expressed as mean ± SE and were analyzed by one-way ANOVA. Post hoc analysis was done by using the Student-Newman-Keuls (SNK) multiple comparison test. *A* value of $P<0.05$ was considered significant.

RESULTS

HE staining

Inflammation change was seen in ileum and mesenteric lymph node in the rats stimulated with STM. There are numerous bacilli in ileum cavity in the infected rats. The structure of the villus of the infected ileum was destroyed (Figure E2), part of epithelial cells were scaled, and many neutrophil, red blood cell and fibroblast infiltrated into the villus. At the same time, secondary lymphoid folliculi appeared in mesenteric lymph node (Figure E4). Figures.E1 and E3 show the normal tissue image of the villus and mesenteric lymph node in saline-treated rat.

FCM

Table 1 shows the percentages and MIFs of CD4+and CD8+ T cells in every group.

Figure1 A shows that subdiagphragmatic vagotomy itself in normal animals had no evident effect on the percentages of CD4+ T cells, but the stimulation of STM itself in sham-operated animals decreased the percentages of CD4+ T cells from 42%±4.5% to 34%±4.9% ($P<0.05$) and after subdiagphragmatic vagotomy the decreased percentages of CD4+ T cells in STM stimulated rats restored from 34%±4.9% to 44%±6.0%, the level of non- STM stimulated rats ($P<0.05$).

Figure1 B shows that subdiagphragmatic vagotomy itself in NS+operation animals decreased MIFs of CD4+ T cells from 6.6±0.6 to 4.9±1.0 ($P<0.05$), indicating the inhibition of subdiagphragmatic vagotomy to CD4+ T cells.

Figure1 C and Figure1 D show that subdiagphragmatic vagotomy itself in normal rats decreased the percentages of CD8+ T cells (from 28%±3.0% to 21%±5.9%, $P<0.05$) as well as MIFs of CD8+ T cells (from 2.9±0.39 to 1.4±0.34, $P<0.05$). STM stimulation itself in sham-operated rats also depressed the percentages of CD8+ T cells (from 28%±3.0% to 21%±5.9%, $P>0.05$) and MIFs of CD8+ T cells (from 2.9±0.39 to 2.1±0.36, $P<0.05$). Subdiagphragmatic vagotomy in STM-challenged rats aggravated the inhibition of STM to the percentages of CD8+ T cells (from 23%±2.0% to 17%±5.8%, $P<0.05$) and MIFs of CD8+ T cells (from 2.1±0.36 to 1.1±0.06, $P<0.05$).

Table 1 Percentages (%) and MIF of CD8+ and CD4+ T cells ($\bar{x}\pm s$)

	NS+sham	NS+vagotomy	STM+sham	STM+vagotomy
CD4	42±4.5	46±4.6	34±4.9[b]	44±6.0[a]
CD4 MIF	6.6±0.6	4.9±1.0[b]	6.8±1.1	6.1±1.0
CD8	28±3.0	21±5.9[b]	23±2.0	17±5.8[a]
CD8 MIF	2.9±0.39	1.4±0.34[b]	2.1±0.36[b]	1.1±0.06[a]

[a]$P<0.05$ *vs* STM+sham; [b]$P<0.05$ *vs* NS+sham

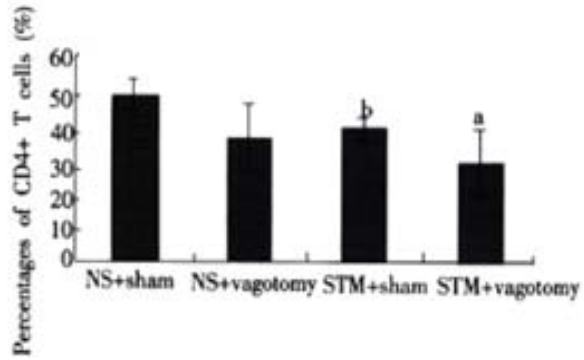

Figure 1A Percentages of blood CD4+ T cells. [a]*P*<0.05 *vs*. STM+sham, [b]*P*<0.05 *vs* NS+sham

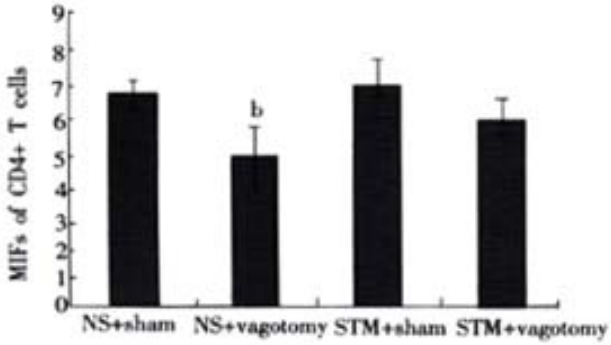

Figure 1B The Mean Intensities of Fluorescence (MIFs) of blood CD4+ T cells. [b]*P*<0.05 *vs* NS+sham

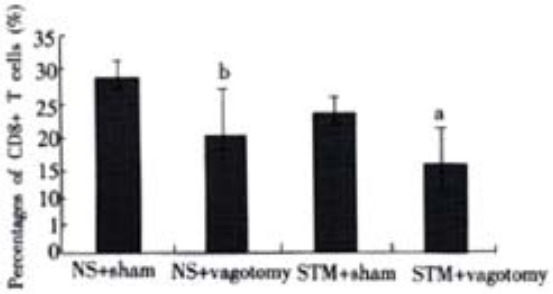

Figure 1C Percentages of blood CD8+ T cells. [a]*P*<0.05 *vs* STM+sham, [b]*P*<0.05 *vs* NS+sham

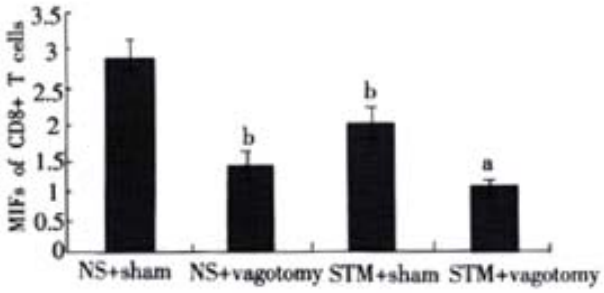

Figure 1D The Mean Intensities of Fluorescence (MIFs) of blood CD8+ T cells. [a]*P*<0.05 *vs* STM+sham; [b]*P*<0.05 *vs* NS+sham

Immunohistochemistry

Table 2 shows the number of Fos-ir cells in PVN and SON in each group. The numbers of Fos-ir cells in PVN and SON of STM+sham-operated rats increased significantly compared with that of NS+sham from 189±41 to 467±62 (*P*<0.05) and from 64±21 to 282±47 (*P*<0.05) individually (Figures.E5, E6, E9, E10). The positive neuron distributed in both magnocellular and parvocellular portions of PVN as well as dorsal and ventral parts of SON. Fos expressions were attenuated in PVN and SON in the rats of STM+vagotomy group compared with that of STM+sham group from 467±62 to 226±45 (*P*<0.05) and from 282±47 to 71±19 (*P*<0.05) individually (Figures. E6, E7, E10, E11), but it was still higher than that of saline-treated animal (189±41 and 64±21 individually). There was no significant changes of Fos expression in NS + vagotomy rats compared with NS + sham rats (Figures.E5, E8, E9, E12).

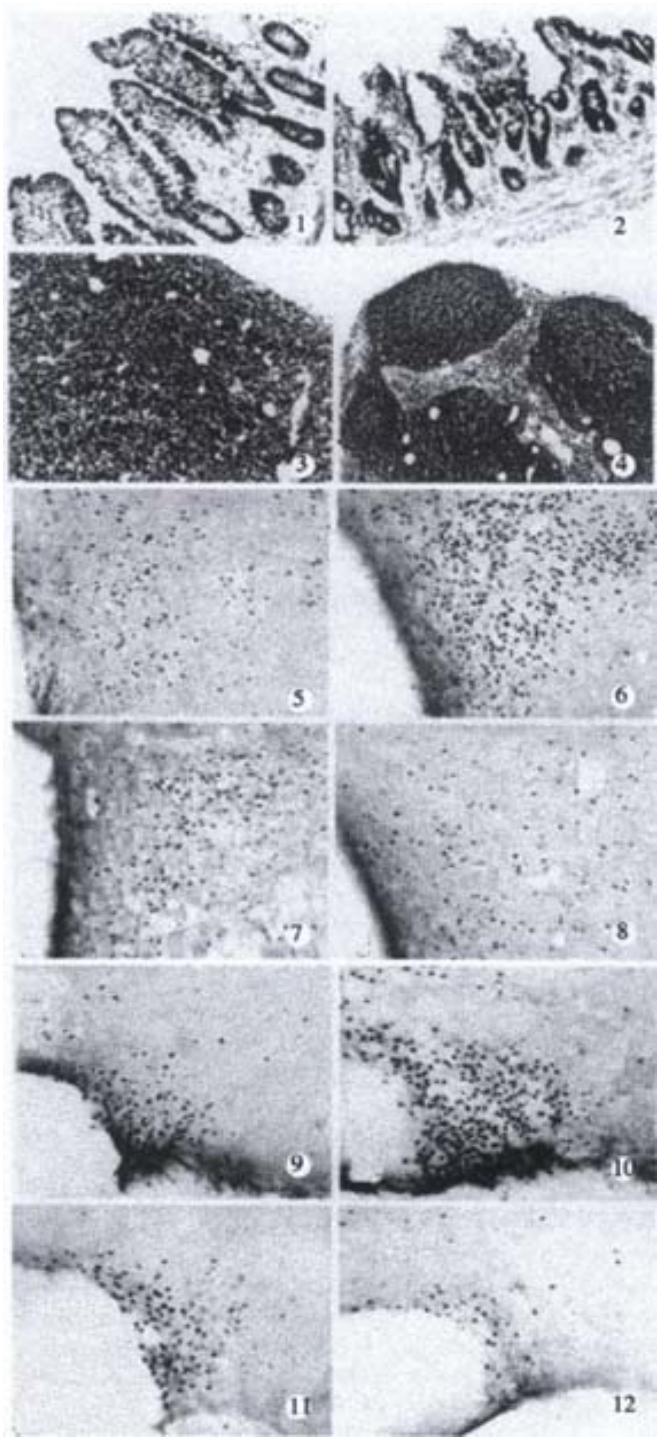

Figure 2 In Figures E 1 and 3 show the normal structures of the villus and mesenteric lymph node in saline-injected rats; 2 and 4 show the villus and mesenteric lymph node in STM-challenged rats. 5 and 9 show Fos expression in PVN and SON respectively in NS +sham rats; 6 and 10 show Fos expressions in PVN and SON respectively in STM +sham rat; 7 and 11 show Fos expressions in PVN and SON respectively in STM +vagotomy rat; 8 and 12 show Fos expressions in PVN and SON respectively in NS +vagotomy rat. ×50

Table 2 Numbers of Fos-ir Cells in PVN and SON ($\bar{x}\pm s$)

	NS + sham	NS + vagotomy	STM + sham	STM + vagotomy
PVN	189±41	131±38	467±62[a]	226±45[b]
SON	64±21	49±22	282±47[a]	71±19[b]

[a]P<0.05 *vs* NS + Sham; [b]P<0.05 *vs* STM + sham

DISCUSSION

More and more evidences have shown that there is a complicated bidirectional inter-relationship between nervous system and immune system[4,16-23]. Immune signals produced during antigen challenge can be transmitted into central nervous system (CNS) and influence the function of the latter. In turn, CNS can modulate the activity of immune system. However, it is still an unsolved problem up to now how the immune signals are transmitted into CNS. Two of hypothesises have been proposed[1-4]: one is through humoral route and the other, via neural pathway. Among the neural pathways the vagus nerve in transferring peripheral immune signals into CNS has been paid more attention to[1-5,24,25]. A large amount of evidences indicate that vagus plays an important role in surveying the peripheral immune information into CNS. For example, subdiagphragmatic vagotomy inhibits a series of brain-mediated responses to peripheral administration of lipopolysaccharide (LPS), IL-1β or TNF-β, such as induction of IL-1β mRNA within mice brain[26,27], activation of hypothalamic corticotropin-releasing hormone neurons and ACTH secretion[28,29], LPS-induced fever in guinea pigs[30], Fos immunoreactivity in primary afferent neurons of the vagus[31], the inhibition of social exploration[32], a monophasic fever[33], the hyperalgesia[34,35] etc. Administration of IL-1β in hepatic portal vein induced afferent discharges of hepatic branch of vagus, but the discharges disappeared in vagotomy rats[36]. Nucleus tractus solitarius lesions attenuated the first fever peak induced by intraperitoneal injection of IL-1β[37].All of the above mentioned experiments indicate that intact vagus is necessary for transmitting the immune information from periphery, especially from peritoneal cavity, to the brain. According to the anatomical structure of vagus, the abdominal organs such as liver, stomach, intestines, lymph node, etc. are innervated mostly by subdiagphragmatic vagus and the vagus contains important visceral sensory afferent fibers from abdominal organs[38,39]. Thus, we conjecture that subdiagphragmatic vagus may play an important role in transmitting the abdominal immune information into the brain and is important in maintaining immune balance.

All the immune challenges used in previous studies were bacterial toxins such as LPS or immune cytokines injected intraperitoneally or intravenously. In this experiment we established a rat model of gastrointestinal tract infection by STM intubation to mimic the natural infection and a subdiagphragmatic vagotomy was performed to further observe the role of vagus in immune signal transmission. According to aetiology, STM can invade intestinal mucosa and largely reproduce, and then further spread into the drained mesenteric lymph nodes and disseminate via the bloodstream[40]. STM is an intracellular Gram-negtive bacterial pathogen that infects both phagocytic and non-phagocytic cells[6,40-43]. It can inhibit the host immune system and cause a range of diseases including enteric fever and gastroenteritis[6]. It has been reported that the depletion of either CD4+ or CD8+ T cells by STM impairs their ability to transfer protective immunity to virulent S. typhimurium[6]. These studies indicate that CD4+ and CD8+ T cells act synergistically to control infection with virulent S. typhimurium[6,44,45]. In our experiment the villus of the infected ileum was destroyed, part of epithelial cells scaled, and the number of neutrophils, red blood cells as well as fibroblasts increased in the villus. At the same time, secondary lymphoid folliculus stimulated with STM emerged in mesenteric lymph nodes. The percentages of CD4+ and CD8+ T cells and MIFs of CD8+ T cells of peripheral blood were all inhibited, which was consistent with the previous reports. These changes induced by STM suggest that our STM infection model was reliable.

The result showed that in NS+sham rats Fos proteins expressed in a few of PVN and SON neurons, which suggests that in normal condition some PVN and SON neurons are active, and may is related to the modulation of routine metabolic activities. After being stimulated with STM the number of Fos-ir cells significantly increased in PVN and SON. It indicated that these cells were activated by STM-challenge. It is well known that CNS, especially hypothalamus, involves in modulation of acute immune reaction[46]. PVN and SON, which are two most important nuclei in hypothalamus related to autonomic function, are mainly composed of three kinds of neurons neurochemically: oxytocinergic, vasopressinergic and CRH neurons[46]. All of these three kinds of neurons can involve in neuroimmunomodulation[46]. Yang *et al*[46] reported that, as the neuroimmunomodulation integrating center, hypothalamic PVN modulates the immune function through three pathways: The first is CRH -ACTH-adrenal cortex axis, the second is oxytocin neuroendocrine pathway, and the third is PVN-spinal cord sympathetic preganglionic projection. Although we can't determine which kind of neurons were activated in this experiment since we did not apply double-labeling technique to identify them, we proposed from the observation of distribution of Fos positive neurons in the subnuclei of PVN and SON that, maybe, all these three kind neurons were activated.

But, how the immune signals are transmitted into the brain is an important and unsolved question. Is it through vagus or humoral pathway, or both of them? What we focused on in the present study was the role of vagus in the sensation and transmission of immune signals to brain. So, we severed subdiagphragmatic vagus to observe whether the Fos expressions in PVN and SON induced by STM infection and the T cell subpopulation were influenced. After subdiagphragmatic vagotomy, Fos expressions in PVN and SON were attenuated. At the same time we found that the decreased percentage of CD4+ T cells in STM-infected rats restored after subdiagphragmatic vagotomy. These results indicate the importance of intact subdiagphragmatic vagus in signaling immune information from abdominal organs to CNS. We tend to conclude from our results that subdiagphragmatic vagus does play a role in transferring immune information into brain during the abdominal inflammatory phase.

However, the detailed mechanism about how vagus nerve senses the immune stimulation and transfers it into electric signal is still not fully understood. It is known that macrophages, dendritic cells, and other immune cells detect and present antigens and respond by releasing proinflammatory mediators, such as IL-1β, IL-6 and TNF-α[23,47,48]. Goehler *et al*[47] found that between the fibers of abdominal vagus there exist immune cells which can produce IL-1β. IL-1β acts to both coordinating the peripheral immune response and signaling the CNS[49]. The globe cells of vagus paraganglia near liver hilus could be stained by biotinylated IL-1 receptor antagonist[50] and by anti rat IL-1 receptor type I antibody[51], which suggested the possibility for vagus to sense the local IL-1. We[51] and others[52] also have reported that the primary sensory neurons in nodose ganglia of vagus contain IL-1 receptor protein and mRNA, which indicates that vagus nerve probably can sense IL-1 directly.

It is necessary to point out that vagus is definitely not the only route for immune signal getting into the brain, since it is found in the present study that although the number of STM stimulation-induced Fos expressed neurons in hypothalamus is attenuated after vagotomy, the number is still higher than that in control. So the humoral pathways or other nerves may also involve in the immune signals transmission in some degree, which still needs further study.

Our results also showed that subdiagphragmatic vagotomy itself decreased the percentages of CD8+ T cells and MIFs of CD4+ and CD8+ T cells, which indicated the importance of intact vagus in maintaining the host immune balance. This is also accordant with our previous study[53]. As we know that CD4+ and CD8+ T cells are necessary in clearing STM[6,54,55]. Vagotomy inhibits the subpopulation of T cells, which is a disadvantage to STM clearance and only aggravate the inhibition to CD4+ and CD8+ T cells induced by STM. How does the vagus influence the phenotype of lymphatic cells? Vagus contains both afferent and efferent fibers innervating abdomen. The former can transmit abdominal information into CNS and the latter innervates some immune organs or immune cells, such as abdominal lymph node. When we cut off subdiagphragmatic vagotomy, on the one hand, the abdominal immune information can't be transmitted into the brain; on the other hand, the brain can't influence the abdominal immune organizations via vagus. We suppose that this is probably the answer.

In summary, subdiagphragmatic vagus is able to signal immune information from abdomen into the brain and intact vagus is necessary in maintaining the host immune balance.

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