Neostigmine modulates tularemia progression in BALB/c mice

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Neostigmine is a pseudo-irreversible inhibitor of enzyme acetylcholinesterase (AChE). We hypothesized link between neostigmine and cholinergic anti-inflammatory pathway via better availability of blood acetylcholine after AChE inhibition and consequent activation of nicotinic acetylcholine receptors. Owing to the expected mechanism of action, we expect significant impact of neostigmine on immunity. In the reported experiment, we used BALB/c mouse model and experimental infection with Francisella tularensis, a causative agent of tularemia. Interferon γ, interleukin 6 and mortality test are done for neostigmine doses of 8.00, 40.0 and 200 µg/kg. We proved significant decrease of both cytokines in course of neostigmine in a dose dependent manner. Neostigmine aggravates mortality caused by tularemia. Owing to the reported results, application of AChE inhibitors to patients suspected with tularemia or similar diseases can be considered as a life endangering therapy.

Key words: Inflammation, tularemia, alzheimer disease, myasthenia gravis, innate immunity.

INTRODUCTION

Neostigmine is a chemical compound with exact IUPAC name 3-(((dimethylamino)carbonyl)oxy)-N,N,N-trimethylbenzenaminiun. Neostigmine is a pseudo-irreversible inhibitor of both cholinesterases: acetylcholinestase (AChE) and butyrylcholinesterase (Pohanka, 2011). Due to the presence of quaternary nitrogen in its structure, neostigmine is not simply crossing the blood-brain barrier and the crossing can occur only in exceptional conditions (Parisi and Francia, 2000). Neostigmine is a frequently used drug. It is well suitable for symptomatic treatment of myasthenia gravis (Mehndiratta et al., 2011) and as a drug for post operative analgesia (Harjai et al., 2010). Besides affecting neuromuscular junctions, neostigmine can meet immune response via cholinergic anti-inflammatory pathway. The cholinergic anti-inflammatory pathway is a regulation on the termination of parasympathetic nervous vagus and α7 nicotinic acetylcholine receptors (nAChR) on macrophages surface (Pavlov and Tracey, 2005). Though neostigmine implication in inflammation has not been extensively researched, amelioration of inflammation can be expected as inhibition of blood AChE that can stop the cholinergic anti-inflammatory pathway (Pohanka, 2011; Pohanka et al., 2011).

We have chosen a model pathogen, Francisella tularensis, a causative agent of tularemia disease. It is an intracellular pathogen and zoonotic agent with fast progression once inoculated into host (Pohanka et al., 2007). For tularemia resolving, organism needs to employ innate immunity and secretion of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and other pro-inflammatory cytokines (Loegering et al., 2006; David et al., 2011). Interferon-γ (IFN-γ) is another important cytokine needed for suppression of tularemia in host (Anderson et al., 2010; Valentino et al., 2011). The present study is aimed at investigating the neostigmine influence on infection pathogenesis. We have chosen tularemia as a model disease for both epidemiological relevance and role of innate immunity to resolve the disease. As interaction of neostigmine with the cholinergic anti-inflammatory pathway can be expected, it is hypothesized that neostigmine is able to change pathogenesis of tularemia. Importance of the inflammatory cytokines for tularemia resolving is undisputed.

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and thus significant implication of neostigmine is a logical but experimentally unconfirmed idea.

MATERIALS AND METHODS

Microorganism

A strain ATCC 29684 of *F. tularensis* LVS was used throughout the experiment. The bacterium was cultivated on McLeod agar supplemented with bovine hemoglobin and Iso VitaleX (Becton-Dickinson, San Jose, CA, USA). The culture was harvested after one day. The cells were suspended into saline solution and washed by centrifugation at 2,000×g for 10 min. Concentration of freshly prepared *F. tularensis* suspension was assessed by cell density meter (WPA, Cambridge, UK) and confirmed one day later by a cultivation test.

Animals

Two months old female BALB/c mice (BioTest, Konarovice, Czech Republic) were used in the experiment. The animals weighted 20±2 g and they were kept in an air conditioned laboratory with temperature 22±2°C, humidity 50±10%, and light period from 7 a.m. to 7 p.m. Food and water were supplied without any obstacles to the animals. The experiment was done in vivarium at the Centre of Biological Defense in Technin (Czech Republic) and it was permitted and supervised by the Ethical Commission of Ministry of Defence, Czech Republic.

Cytokines investigation

In the first part of the experiment, cytokine markers were investigated. In a total, 64 mice were divided into 8 groups of 8 animals each. The animals were exposed to saline, neostigmine (neostigmine bromide, 98% purity, purchased from Sigma-Aldrich, St. Louis, MO, USA), infected with tularemia or received co-exposure to neostigmine and infection with tularemia. The individual groups received the following:

1. Controls- 100 µl of saline only.
2. Neostigmine 8.00 µg/kg.
3. Neostigmine 40.0 µg/kg.
4. Neostigmine 200 µg/kg.
5. *F. tularensis*.
6. *F. tularensis* + neostigmine 8.00 µg/kg.
7. *F. tularensis* + neostigmine 40.0 µg/kg.
8. *F. tularensis* + neostigmine 200 µg/kg.

*F. tularensis* was given subcutaneously. 100 µl of the suspension containing 10⁶ CFU/ml was given as a saline solution in the right rear limb. Neostigmine was dissolved in saline as well. It was given in the second (left) rear limb in an amount of 100 µl containing 0.16, 0.80 or 4.0 µg of neostigmine pro toto (that is 8, 40 and 200 µg/kg doses). The neostigmine doses were chosen in order to be close to human therapeutic dose (the median dose) and then the five times higher and lower doses (Lederer et al., 2010). The upper dose of neostigmine was chosen in order to be close to median lethal dose (Starč et al., 1997). The animals were sacrificed under CO₂ anesthesia three days after the beginning of the experiment. Blood was collected into heparinized syringe and plasma was received by centrifugation of the blood at 1,000×g for 5 min. IL-6 and IFN-γ concentrations were assessed by an indirect enzyme linked immuno-sorbent assay (ELISA). Murine IL-6 Eli-pair kit and a Mouse IFN-γ Eli-pair kit (Abcam, Cambridge, MA, USA) were purchased and applied for the assay purposes.

Mortality test

100 mice were divided into five groups and they received the following:

1. Controls, 100 µl of saline only.
2. *F. tularensis*.
3. *F. tularensis* + neostigmine 8.00 µg/kg.
4. *F. tularensis* + neostigmine 40.0 µg/kg.
5. *F. tularensis* + neostigmine 200 µg/kg.

*F. tularensis* and neostigmine were given in the same way as written in the previous chapter in a dose of 10⁶ CFU/ml suspension containing 10⁷ CFU/ml in saline. Animals in the mortality test were kept for two weeks and their shape and demise were observed.

Statistical analysis

The experimental data were processed using Origin 8 (Origin Lab Corporation, Northampton, MA, USA). Significances of alterations between the tested groups were estimated using one-way analysis of variance with Bonferroni test for probabilities levels p=0.05 and p=0.01 for 8 specimens in one group.

RESULTS

The cytokines IL-6 and IFN-γ are depicted as shown in Figures 1 and 2. The IL-6 was not altered in course of neostigmine when the animals were not infected with tularemia. The tularemia caused significant (p=0.01) increase of IL-6 in the mice (Figure 1). Administration of neostigmine into the infected animals caused demise of IL-6. Effect of the lowest dose of neostigmine was insignificant. On the other hand, the doses 40.0 and 200 µg/kg altered IL-6 level to value insignificant to the controls. The highest dose of neostigmine caused significant (p=0.01) demise of IL-6 in tularemia infected mice compared to the infected animals with no administration of neostigmine. Alterations of IFN-γ in course of neostigmine were similar to the IL-6. We proved no significant link between IFN-γ level and neostigmine dose in the health animals. However, tularemia infected mice had decreased IFN-γ level in a disproportional dose-response shape (Figure 2). In tularemia infected mice, the two upper doses: 40.0 and 200 µg/kg caused decrease of IFN-γ to level insignificant to controls. Mortality test is depicted as shown in Figure 3. Mortality in the course of tularemia reached 40% (60% survival rate). Once neostigmine administered to animals suffered from tularemia, mortality increased in a dose response manner from 50 (neostigmine 8.00 µg/kg) to 65% (neostigmine 200 µg/kg).

DISCUSSION

Both innate and adaptive immunity are necessary for
resolving of tularemia. Macrophages in combination with T cells have key role in the immunity response to tularemia (Conlan et al., 1994). Deficiency in the T cell mediated immunity abrogates ability to resolve tularemia (Ray et al., 2009). On the other hand, both immunoglobulins M and G are participating in murine response to tularemia as well (Pohanka, 2007).

Chemotherapy implicated in innate immunity can result in significant pathogenesis of tularemia. Once innate immunity ameliorated, aggravation of tularemia can be expected. We proved that neostigmine significantly decreases both IL-6 and IFN-γ in tularemia infected mice. It can be interpreted that neostigmine acts as an anti-inflammatory drug. The data are quite plausible as both
cytokines decreased in a dose dependent manner. Moreover, the mortality aggravation confirms the anti-inflammatory action when role of innate immunity in fighting with tularemia is considered. We infer that neostigmine meets the cholinergic anti-inflammatory pathway and in that way it influences immune system and probability to survive tularemia. The cholinergic anti-inflammatory pathway can ameliorate inflammation via α7 nAChR located on macrophages (Bernik et al., 2002; Zitnik, 2011).

Activation of the cholinergic anti-inflammatory pathway is desirable in sepsis when it may allow increase of surviving probability (Song et al., 2008; Parrish et al., 2008). Beside the sepsis treatment, activation of the pathway is a good tool for amelioration of inflammatory health complications such as post-operative ileus (The et al., 2011). Current effort in anti-inflammatory therapy is devoted to introduction of novel compounds and preliminary studies are promising (Lee and Park, 2011; Rosas-Ballina et al., 2009). The inflammation can be regulated by: Impact on acetylcholine receptors and enzyme AChE as the neurotransmitter acetylcholine which can become better available once AChE does not split it (Pohanka, 2012). Though stimulation of the cholinergic anti-inflammatory pathway is desirable in some cases like the mentioned sepsis and chronic inflammation, we infer that it is not suitable in tularemia and similarly progressing diseases. Importance of inflammatory processes for resolving of tularemia was independently confirmed in many experiments. Moreover, the bacterium has ability to protect itself by blocking proinflammatory cytokines release (Periasamy et al., 2011). Due to the facts of the meaning of inflammatory therapy, anti-inflammatory therapy can be expected as not convenient for the disease. Neostigmine pseudo-reversibly inhibits AChE; however, it is not a well crossing blood brain barrier and so it is preferably targeting blood AChE and AChE in neuromuscular junctions. Central nervous system is not affected by neostigmine in an extensive scale (Pohanka, 2011). We hypothesize that after inhibition of the blood AChE by neostigmine, increasing level of blood acetylcholine activates the cholinergic anti-inflammatory pathway. Though we have no direct evidence of neostigmine implication in the cholinergic anti-inflammatory pathway, the proved decreases of IFN-γ and IL-6 in an indirectly proportionally dose response manner confirm the hypothesis when immunological meaning of the markers is considered (Golicnik et al., 2012). Aggravated mortality in course of tularemia is another plausible proof confirming the hypothesis as well.

Owing to the results, administration of neostigmine should be carefully considered in patients suspected from early stages of tularemia or similarly acting infectious diseases. On the other hand, neostigmine application could be beneficial in sepsis as it abrogates immunity. The finding is supported by experiments done on experimental sepsis model with a wide scale of cholinesterases inhibitors (Hofer et al., 2008).

Neostigmine was investigated by Akinci et al. (2005) in...
mice suffering from endotoxemia. The authors reported no effect of neostigmine in doses of 0.1 mg/kg but significant decrease of interstitial inflammation for a dose of 0.3 mg/kg and overall results are ambiguous. In another study, neostigmine was found able to attenuate TNF-α expression in the heart with pressure overload (Freeling et al., 2008). We expect that similar potency to influence tularemia like neostigmine has other AChE inhibitors used in current therapy of Alzheimer’s disease, myasthenia gravis and some carbamate pesticides such as rivastigmine, pyridostigmine, carbofuran etc.

The preliminary results from here described experiment and the above report confirmed the idea. The dose of neostigmine (40.0 µg/kg) corresponds with the therapeutic dose in humans (Lederer et al., 2010). Thus the effects proved here for the median applied dose can be simply extrapolated to the humans treated with neostigmine or structurally similar drugs. The results do not plausibly confirm that cholinergic anti-inflammatory pathway is the main target of neostigmine involved in the effect of tularemia pathogenesis. On the other hand, implication of neostigmine in modulation of inflammation is proved and the cholinergic anti-inflammatory pathway seems to be the most probable target of the neostigmine effect.

Conclusion

Neostigmine aggravates progression of tularemia via abrogation of innate immunity. Owing to the results, application of AChE inhibitors to patients suspected with tularemia or similar diseases can be considered as a life endangering therapy. Special attention should be given to drugs for therapy of myasthenia gravis or Alzheimer’s disease.

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