ORIGINAL ARTICLE

Tacrine alters antibodies level in *Francisella tularensis*-infected mice

Miroslav Pohanka [1,2], Oto Pavlis [1,3], Jiri Pikula [4]

1 Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
2 Karel English College in Brno, Brno, Czech Republic
3 Centre of Biological Defence, Techonin, Czech Republic
4 Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

Correspondence to: Assoc. Prof. Miroslav Pohanka, PhD.
Faculty of Military Health Sciences, Brno, University of Defence
Trebesska 1575, 50001 Hradec Kralove, Czech Republic
TEL: +420973253091; E-MAIL: miroslav.pohanka@gmail.com

Submitted: 2013-06-21 Accepted: 2013-08-30 Published online: 2013-11-10

Key words: **acetylcholinesterase; immune reactivity; Francisella tularensis; neuroimmunology; Alzheimer disease**

Neuroendocrinol Lett 2013; **34**(Suppl.2):134–137 **PMID:** 24362106 NEL341013A21

Abstract

OBJECTIVES: Tacrine is an inhibitor of acetylcholinestrase (AChE) formerly used to treat cognitive impairment of Alzheimer disease. In previous works, we have shown that inhibitors of AChE can modulate innate immunity responses. In the present study we focused on modulation of adaptive immunity represented by production of antibodies. It is hypothesized that the cholinergic anti-inflammatory pathway is a common mechanism how inhibitors of AChE can influence immunity. Here, tularemia is used as a model disease for experimental purposes.
DESIGN: A total of 64 BALB/c mice were divided into eight groups. The animals received a dose of tacrine 0.1–0.5 mg/kg with combination of saline or inoculum of Francisella tularensis. The doses of tacrine were derived from clinical trials. The animals were sacrificed after three days and total antibodies in plasma and bacterial burden in the liver were measured.
RESULTS: Tacrine did not alter the antibodies level in non-infected animals. Antibodies levels of infected animals administered tacrine were reduced in a dose response manner. Tacrine also caused an increase in total bacteria numbers in the liver.
CONCLUSIONS: Tacrine significantly suppressed adaptive immunity represented by the ability of the organism to produce antibodies. We infer that tacrine can modulate the cholinergic anti-inflammatory pathway by a mechanism based on inhibition of blood AChE followed by higher availability of acetylcholine. The anti-inflammatory pathway is then stimulated and the body is not able to simply resolve antigen. Application of an AChE inhibitor during infectious diseases can have detrimental consequences for the immune system.

Abbreviations:
AChE - acetylcholinesterase
nAChR - nicotinic acetylcholine receptor

INTRODUCTION

The cholinergic nervous system is a basal excitation pathway comprised both in the peripheral and central nervous system. In this pathway, acetylcholine is a common compound acting as a neurotransmitter allowing the spread of the signal between two neurons or between a neuron and an effector cell. The role of acetylcholine as a mediator is emphasized by a number of cells and tissues. Apart from the nervous system, acetylcholine receptors can be found in endothelium and cells of the immune system (Wessler & Kirkpatrick 2008).

Acetylcholine plays a significant role in the immune system. In the peripheral part of the nervous system, it acts as a mediator of the cholinergic anti-inflammatory pathway. The cholinergic anti-inflammatory pathway consists of *nervus vagus* terminations in the bloodstream, macrophages having α7 nicotinic acetylcholine receptor (α7 nAChR), and inflammatory cytokines such as tumour necrosis factor α and high-mobility group protein 1 (Rosas-Ballina & Tracey 2009; Huston 2012; Pohanka 2012b). The anti-inflammatory pathway has a significant role in the regulation of inflammatory reactions and it represents a link between the nervous and immune systems. The pathway can be modulated by either agonizing / antagonizing on the α7 nAChR or by inhibition of erythrocyte-associated AChE that causes better availability of the neuromediator acetylcholine (Pavlov *et al.* 2007; Pohanka 2012a,b, 2013).

The fact that compounds inhibiting AChE could cause alteration of immune reactivity is quite interesting because the inhibitors have wide pharmacological (Pohanka 2011b). The current experiment aimed at immunity modulation by tacrine (9-amino-1,2,3,4-tetrahydroacridine) during tularemia disease. Tacrine is an AChE inhibitor binding into the peripheral anionic site. To a lesser degree it can also inhibit butyrylcholinesterase (Ahmed *et al.* 2006; Pohanka 2012a). Until withdrawal from use due to adverse side effects, tacrine was used as a cognitive enhancer in patients suffering from Alzheimer disease (Davis *et al.* 1992; Alfirevic *et al.* 2007). Tularemia is a disease caused by the gram negative bacterium *Francisella tularensis*. Every organism needs both innate and adaptive immunity to handle diseases (Ellis *et al.* 2002; Parmely *et al.* 2009). We hypothesize that tacrine can modulate immune responses to infections such as tularemia.

MATERIAL AND METHODS

Bacterium

The bacterium *F. tularensis* LVS (ATCC 29684) was chosen for the experiment as a strain pathogenic for rodents. The bacterium was cultivated on McLeod agar supplemented with bovine blood haemoglobin (Sigma-Aldrich, St. Louis, MO, USA) and Iso VitaleX (Becton-Dickinson, San Jose, CA, USA). The agar plates were kept at 37 °C for one day. After that, the bacterial colonies were scraped from the plate, washed by centrifugation at 2,000 ×*g* for 5 minutes, and suspended in saline up to 3×10^6 CFU/ml. The exact concentration of bacteria in the suspension was confirmed by cultivation test one day later.

Experimental design

Conventional female BALB/c mice were used in the experiment. A total of 64 animals were divided into eight groups kept separately. At the start of the experiment, the body mass of eight-week-old animals was 21±1 g. The whole experiment was done in accredited vivarium of the Centre of Biological Defence in Techonin (Czech Republic) and supervised by the ethical committee of Ministry of Defence (Czech Republic). Temperature in the vivarium was 22±2 °C, humidity 50±10%, and photoperiod of 12 hours a day.

F. tularensis suspension and/or tacrine in the amount of 100 µl were applied subcutaneously into either left or right pelvic limb. Saline was used as a control in the case when the aforementioned solutions were not applied. The animals in the tested groups received the following:

group 1: double dose of saline;
group 2: 0.02 mg/kg of tacrine on day 1 and 2 of the experiment;
group 3: 0.1 mg/kg of tacrine on day 1 and 2;
group 4: 0.5 mg/kg of tacrine on day 1 and 2;
group 5: a dose of the *F. tularensis* suspension;
group 6: a dose of the *F. tularensis* suspension and 0.02 mg/kg of tacrine on day 1 and 2;
group 7: a dose of the *F. tularensis* suspension and 0.1 mg/kg of tacrine on day 1 and 2;
group 8: a dose of the *F. tularensis* suspension and 0.5 mg/kg of tacrine on day 1 and 2.

The animals were sacrificed by cardiac puncture under CO_2 narcosis on day 3 of the experiment. Blood was collected into standard disposable plastic tubes with absorbed lithium heparin (Dialab, Prague, Czech Republic). Plasma was separated by centrifugation at 1,000 × g for 5 minutes. The livers were separated from the cadavers and they were mechanically homogenized by repeated passing through a plastic net with hole size 1 mm^2. The homogenate was cultured and number of viable bacteria was calculated.

Enzyme Linked Immuno-Sorbent Assay (ELISA)

Standard 96 well microplates Maxisorp (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were used for the assay. A total 100 µl of ten times diluted plasma were placed per one well and incubated at room temperature overnight. Wells were washed by phosphate buffered saline (PBS) and blocked by 100 µl of 0.1% (w/v) gelatine for one hour. After repeated washing by PBS, peroxidase-labelled antibodies against mouse immunoglobulins (Sigma-Aldrich) was applied in dilution 1:10,000 and incubated at 37 °C for another 60 minutes. The wells were washed by PBS and then PBS with

tween. Peroxidase mediated reaction was visualised by addition of 3,3´,5,5´-tetramethylbenzidine and 5 mmol/l H_2O_2 and left for 15 minutes. Finally, the reaction was stopped by addition of 2 mol/l sulfuric acid in the amount of 100 μl and optical density was measured at 650 nm.

Statistical analysis

Experimental data were processed in Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA). One-way ANOVA with Bonferroni test was used to examine statistical significance. All tests were considered statistically significant and highly significant when resulting in values of $p<0.05$ and $p<0.01$, respectively.

RESULTS AND DISCUSSION

No animal died prior to euthanasia in this experiment. Symptoms of disease such as bristled fur developed second day of the experiment. The clinical manifestation reached maximal level on third day of the experiment and it was in compliance with expectations known from literature and previous experiments (Hepburn & Simpson 2008; Nigrovic & Wingerter 2008; Bandouchova *et al.* 2009; Pohanka & Pavlis 2012). The used doses of tacrine were derived from clinical trials in Alzheimer disease patients and covered the formerly used human therapeutic doses of 0.1–0.5 mg/kg (Davis *et al.* 1992; Knapp *et al.* 1994). In compliance with the expectation, tacrine alone did not cause any manifestation of illness in the mice.

Figures 1 and 2 show total levels of bacteria in the liver and total levels of immunoglobulins in plasma. There is a growing number of bacteria in the liver as the dose of tacrine increased (Figure 1). The increase was significant even in the lowest dose of tacrine. We did not find any bacteria in non-infected animals. Considering the bacterial burden, tacrine makes tularemia worse. The total number of bacteria increased from 3.64×10^5 CFU in the infected only to 2.38×10^6 CFU in the infected and treated with tacrine by the dose of 0.1 mg/kg. The increase was approximately 6.5-fold. Administration of tacrine or a similarly acting compound would result in a serious complication with fatal consequences in infected individuals. Results on the total antibodies level (Figure 2) are in compliance with the findings concerning bacterial burden. The level of antibodies in the controls and tularemia infected animals corresponded to the expectations (Koskela & Salminem 1985; Pohanka 2007). In the non-infected animals, tacrine had no effect. However, the compound significantly suppressed production of antibodies in the infected animals. The effect was dose-dependent. The suppression of antibodies production is probably one of the reasons why tacrine worsens the course of tularemia.

Tacrine is a reversible inhibitor of AChE with no other significant biological targets (Pohanka 2011a). The findings reported here are in good compliance with previous experiments. Tacrine was shown to be effective in suppression of immune responses through interleukin 6 and interferon γ (Pohanka & Pavlis 2013). Similar findings were proved for neostigmine and tularemia (Pohanka & Pavlis 2012). Neostigmine inhibits both AChE and butyrylcholinesterase by a pseudo-irreversible mechanism of inhibition (Pohanka 2013). Owing to immunomodulation, a similar or the same mechanism of action can be anticipated for both tacrine and neostigmine. Unlike neostigmine, tacrine can also cross the blood-brain barrier (Jogani *et al.* 2007). Immunomodulation in the central nervous system is probable. However, the fact that tacrine (an inhibitor to

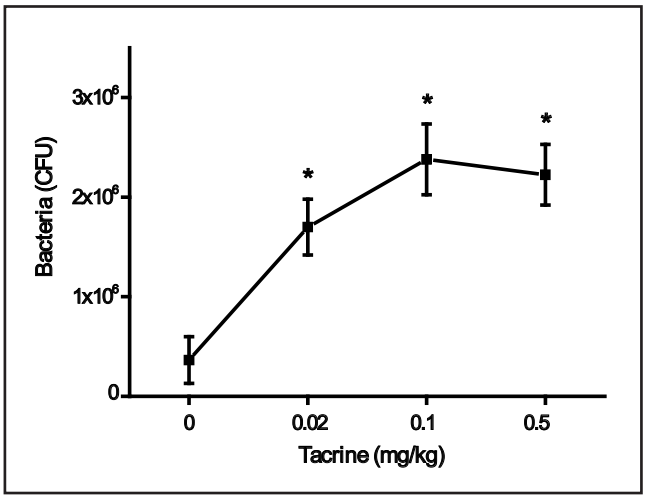

Fig. 1. Total burden of *F. tularensis* bacteria in the infected mice (groups 5-8). Error bars indicate standard deviation. Asterisk symbolizes a significant difference (p=0.05) against the infected animals treated with saline only

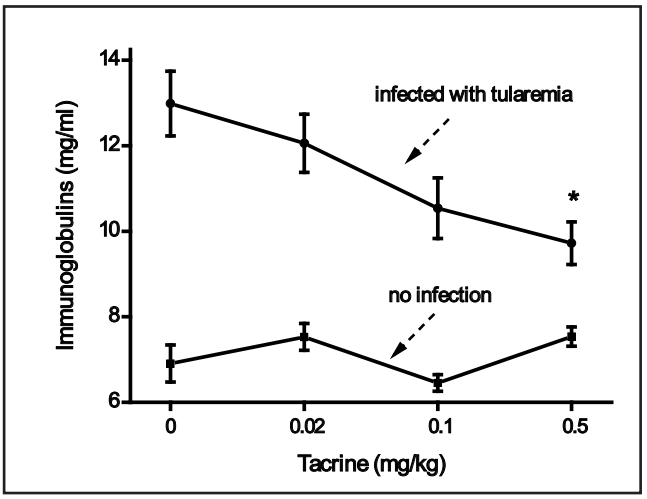

Fig. 2. Total immunoglobulin in plasma of the tested mice. Error bars indicate standard deviation. Asterisk symbolizes a significant difference (p=0.05) against the infected animals treated with saline only.

both the central and peripheral nervous system AChE) and neostigmine (an inhibitor of the peripheral AChE only) act in a similar way suggests immunomodulation mediated via the peripheral nervous system.

Examining the initial hypothesis, it can be stated that tacrine can suppress the adaptive immunity. Probably as a consequence to this suppression, the innate immune responses are also decreased in the presence of tacrine (Pohanka & Pavlis 2013). We infer that tacrine inhibits AChE in blood and in this way causes higher availability of acetylcholine. The cholinergic anti-inflammatory pathway becomes stimulated as a consequence of accumulated acetylcholine (Pohanka 2012b). Both innate and adaptive immunity are necessary for the defence against *F. tularensis* and impairment of the innate immunity has fatal consequences for the adaptive immunity (Bosio 2011; Zarrela *et al.* 2011). Regarding these results, we infer that inhibitors of cholinesterases stimulate the cholinergic anti-inflammatory pathway and interfere with the protection against infectious diseases.

CONCLUSIONS

Inhibitors of AChE can modulate the immune system via the cholinergic anti-inflammatory pathway. Higher availability of acetylcholine in blood is the reason for this effect. The finding has significant relevance because inhibitors of AChE have important pharmacological and toxicological applications and modulation of the immune system can be a complication. On the other hand, modulation of the immune system can explain side effects of the inhibitors with unknown mechanisms of initiation and regulation.

ACKNOWLEDGMENTS

The study was supported by institutional research funds of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The Ministry of Education, Youth and Sports of the Czech Republic is gratefully acknowledged for project LH11023.

REFERENCES

1 Ahmed M, Rocha JB, Correa M, Mazzanti CM, Zanin RF, Morsch AL, et al (2006). Inhibition of two different cholinesterases by tacrine. Chem Biol Interact. **162**: 165–171.
2 Alfirevic A, Mills T, Carr D, Barratt BJ, Jawaid A, Sherwood J, et al (2007). Tacrine-induced liver damage: an analysis of 19 candidate genes. Pharmacogenet Genomics. **17**: 1091–1100.
3 Bandouchova H, Sedlackova J, Pohanka M, Novotny L, Hubalek M, Treml F, et al (2009). Tularemia induces different biochemical responses in BALB/c mice and common voles. BMC Infect Dis. **9:** doi:10.1186/1471-2334-9-101
4 Bosio CM (2011). The subversion of the immune system by francisella tularensis. Front Microbiol. **2**: 9.
5 Davis KL, Thal LJ, Gamzu ER, Davis CS, Woolson RF, Gracon SI, et al (1992). A double-blind, placebo-controlled multicenter study of tacrine for Alzheimers-disease. N Engl J Med. **327**: 1253–1259.
6 Ellis J, Oyston PCF, Green M, Titball RW (2002). Tularemia. Clin Microbiol Rev. **15**: 631–646.
7 Hepburn MJ, Simpson AJH (2008). Tularemia: current diagnosis and treatment options. Expert Rev Anti-Infect Ther. **6**: 231–240.
8 Huston JM (2012). The vagus nerve and the inflammatory reflex: wandering on a new treatment paradigm for systemic inflammation and sepsis. Surgical Infect. **13**: 187–193.
9 Jogani VV, Shah PJ, Mishra P, Mishra AK, Misra AR (2007). Nose-to-brain delivery of tacrine. J Pharm Pharmacol. **59**: 1199–1205.
10 Knapp MJ, Gracon SI, Davis CS, Solomon PR, Pendlebury WW, Knopman DS (1994). Efficacy and safety of high-dose tacrine – a 30 week evaluation Alzheimer Dis Assoc Dis. **8**: S22–S31
11 Koskela P, Salminem A (1985). Humoral immunity against Francisella tularensis after natural infection. J Clin Microbiol. **22**: 973–979
12 Nigrovic LE, Wingerter SL (2008). Tularemia. Infect Dis Clin North Am. **22**: 489–504.
13 Parmely MJ, Fischer JL, Pinson DM (2009). Programmed cell death and the pathogenesis of tissue injury induced by type A Francisella tularensis. FEMS Microbiol Lett. **30**: 1–11.
14 Pavlov VA, Ochani M, Parrish WR, Rosas-Ballina M, Ochani K, Al-Abed Y, et al (2007). The anti-inflammatory efficacy of galantamine is dependent on the integrity of the cholinergic anti-inflammatory pathway. Shock. **27**: 23–23.
15 Pohanka M (2007). Evaluation of immunoglobulin production during tularaemia infection in BALB/c mouse model. Acta Vet Brno. **76**: 579–584.
16 Pohanka M (2011a). Alzheimer's disease and related neurodegenerative disorders: implication and counteracting of melatonin. J Appl Biomed. **9**: 185–196.
17 Pohanka M (2011b). Cholinesterases, a target of pharmacology and toxicology. Biomed Pap. **155**: 219–229.
18 Pohanka M (2012a). Acetylcholinesterase inhibitors: a patent review (2008 - present). Expert Opin Ther Pat. **22**: 871–886.
19 Pohanka M (2012b). Alpha7 nicotinic acetylcholine receptor is a target in pharmacology and toxicology. Int J Mol Sci. **13**: 2219–2238.
20 Pohanka M (2013). Cholinesterases in biorecognition and biosensor construction, a review. Anal Lett., In press. DOI: 10.1080/00032719.2013.
21 Pohanka M, Pavlis O (2012). Neostigmine modulates tularemia progression in BALB/c mice. Afr J Pharm Pharmaco. **6**: 1317–1322.
22 Pohanka M, Pavlis O (2013). Tacrine can suppress immune response to tularemia in BALB/c mouse model J Appl Biomed. **11**: 187–193.
23 Rosas-Ballina M, Tracey KJ (2009). Cholinergic control of inflammation. J Intern Med. **265**: 663–679.
24 Wessler I, Kirkpatrick CJ (2008). Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Brit J Pharmacol. **154**: 1558–1571.
25 Zarrela TM, Singh A, Bitsaktsis C, Rahman T, Sahay B, Feustel PJ, Gosselin EJ, et al(2011). Host-adaptation of Francisella tularensis alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS One. **6**: e22335.