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HI-6 modulates immunization efficacy in a BALB/c mouse model

Miroslav Pohanka[a,b,*]

[a] *Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 50001 Hradec Kralove, Czech Republic*
[b] *Karel Englis College in Brno, Sujanovo namesti 356/1, 60200 Brno, Czech Republic*

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ABSTRACT

HI-6 is used as an antidote to nerve agents. It can also act as an antagonist to acetylcholine receptors (AChRs) including the nicotinic receptor, α 7 nAChR which is involved in regulating the immune response through macrophages. This experiment investigated the efficacy of HI-6 to regulate the immune response. Laboratory BALB/c mice received HI-6 and/or keyhole limpet hemocyanin (KLH) as an antigen. Antibody production was investigated after either 21 or 65 days when either single or repeated dose of antigen was applied. We confirmed that HI-6 significantly improved vaccination efficacy when KLH was given in a dose of 1 mg/kg. The effect was dose dependent. A combination of HI-6 and KLH produced a vaccination of almost the same efficacy as that for Freund's complete adjuvant. The findings point at the suitability of HI-6 for improving vaccination efficacy at the level of immunity regulation by the nervous system.

1. Introduction

Asoxime, also known as HI-6 and chemically characterized as 4-carbamoyl-1-[((2-[(*E*)-(hydroxyimino)methyl]pyridinium-1-yl)methoxy)methyl]pyridinium dichloride, was introduced as an antidote to nerve agents such as sarin, tabun, soman and VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) (Cetkovic et al., 1984; Kepner and Wolthuis, 1978; Koplovitz and Stewart, 1994), some of the most lethal chemical warfare agents. They are also easily disseminated and highly toxic. Their common mechanism of action is irreversible inhibition of acetylcholinesterase (AChE), the enzyme that terminates cholinergic neurotransmission. Acetylcholine receptors (AChRs) become overstimulated with life threatening consequences (Furtado et al., 2012). HI-6 is able to split the organophosphorus moiety from active site of the enzyme and the AChRs are then protected from the overstimulation (Pohanka, 2011, 2012a). HI-6 is available as a drug for military purposes as injection, tablet or plaster (Kassa, 2002). Though conventional use of nerve agents is prohibited by international conventions, the toxins can be misused for terrorist purposes and search of effective treatment is still ongoing in many countries (Chalela and Burnett, 2012).

Though HI-6 is an antidote for the recovery of AChE activity, its effect is more complex. It can act as both a reversible inhibitor of AChE and antagonist to AChRs including α 7 nAChR which is involved in regulating inflammation through the cholinergic anti-inflammatory pathway (Pohanka, 2012b). Due to the presence of two quaternary nitrogens in one HI-6 molecule, the compound has limited ability to cross the blood brain barrier, making it more significant in the peripheral

* Tel.: +420 9732530910; fax: +420 9732530910.
E-mail address: miroslav.pohanka@gmail.com
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nervous system, at nerve terminations and in blood than in the CNS (Dadparvar et al., 2011).

The experiment described below addresses HI-6 involvement in regulating immunity. To recapitulate, HI-6 is able to modulate the cholinergic anti-inflammatory pathway associated with macrophages. This pathway plays a well-known regulatory role during systemic inflammation and sepsis (Huston, 2012). On the other hand, it has an effect on control of antigen processing and presentation including Th1/Th2 lymphocyte balance. A role in regulating antibody production can be assumed as well (Forsythe, 2012). The question we asked was whether HI-6 would be suitable for an improved vaccination against the nerve agents mentioned.

2. Experimental

2.1. Experiment on animals

Two month old female mice BALB/c (Velaz, Unetice, Czech Republic) were used. For the whole experiment, the mice were kept in an air conditioned laboratory with regulated conditions: temperature 22 ± 2 °C, humidity 50 ± 10%, and light period from 7 a.m. to 7 p.m. Food and water were provided ad libitum. The mice weighed 21 ± 3 g at the outset. The experiment was approved by the Ethics Committee of the Faculty of Military Health Sciences, University of Defense (Hradec Kralove, Czech Republic).

In total, 320 animals were divided into 40 experimental groups of 8 animals per group. Keyhole limpet hemocyanin (KLH), HI-6 in chloride salt and Freund's complete adjuvant were purchased from Sigma–Aldrich (St. Louis, MO, USA). KLH and HI-6 were dissolved in saline solution. All liquids were applied in a volume of 100 μl intramuscularly into the rear limb. The composition of the groups is shown in Table 1. The animals were sacrificed in carbon dioxide narcosis by cutting the carotid. Blood was collected directly into tubes with lithium heparin (Dialab, Prague, Czech Republic). The freshly collected blood was centrifuged at 1000 × *g* for 5 min and plasma was poured into a separate tube and stored at −80 °C until assay.

2.2. Enzyme-linked immunosorbent assay (ELISA) for antibodies in serum

Standard 96-well microplates Maxisorp (Nunc; Thermo Fisher Scientific; Waltham, MA, USA) were used for the assay. Anti-mouse polyvalent immunoglobulin (IgG, IgA, IgM) peroxidase conjugate developed in goats was purchased from Sigma–Aldrich. For the standard ELISA we used the following steps: KLH solution in PBS or saline (10 μg/ml) in a volume 100 μl per well was mixed and incubated overnight at 4 °C. After the incubation, the wells were washed twice using 400 μl of PBS with Tween 20. The wells were blocked with 100 μl of 5% (w/w) bovine albumin for 2 h. After blocking, 100 μl of one hundred times diluted plasma were injected per well and incubated for 90 min at 37 °C and the wells were washed by PBS. After incubation, the wells were washed twice using 400 μl of PBS with Tween 20. After washing, 100 μl of secondary antibody against mouse immunoglobulins labeled with horse radish peroxidase (HRP) were applied for another 60 min at 37 °C. The secondary antibody was diluted 10,000 times (standard antibody solution from Sigma–Aldrich, St Louis, MO, USA). The wells were washed three times using 400 μl of PBS with Tween 20. A fresh solution of 0.5 mg/ml 3,3′,5,5′-tetramethylbenzidine and 5 mM H_2O_2 – 100 μl was added for 15 min while the whole plate was kept in the dark. The reaction was stopped with 100 μl per well of 2 mol/l H_2SO_4. The optical density was measured by the ELISA reader Sunrise (Salzburg, Austria) at 650 nm. Wells with captured albumin (20 μl; 5 mg/ml) were used as a negative control and the value was subtracted from value of the examined samples.

2.3. ELISA for interleukin 6 (IL-6)

IL-6 was assayed using a commercial ELISA kit from Abcam (Cambridge, MA, USA). The kit was processed in compliance with the attached instructions using the polystyrene 96-well microplates mentioned (Gama, Ceske Budejovice, Czech Republic) and the microplate reader.

2.4. Statistical processing of the experimental data

The data were processed in Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA) software using a one-way ANOVA with Bonferroni test and a probability level $P = 0.05$.

3. Results and discussion

No alteration in the mice behavior was revealed and the animals had no pathological manifestation. Doses of test substance were chosen to be safe. The KLH doses for mice were chosen to be sufficiently immunogenic and were derived from the cited papers (Gesheva et al., 2011; Kiraly et al., 2011). The doses for HI-6 were under 2% and 0.2% of median lethal dose (15.6 and 1.56 mg/kg) for BALB/c mice. Freund's complete adjuvant dose was chosen to be safe, avoiding pain, necrosis and other unwanted effects (Hessler et al., 1988; Stills, 2005; Toth et al., 1989). The time for euthanasia was chosen as a suitable interval for antibody production (Pohanka, 2007). In a shorter interval, IgM antibodies with lower specificity could be expected. 21 days after beginning experiments, specific antibodies instead of the antibodies with lower specificity are expressed in the BALB/c mouse model (Pohanka, 2007).

IL-6 in plasma did not exceed 40 pg/ml in any of the tested groups. It was chosen as a marker for revealing an inflammatory reaction (Okada et al., 2001). The cytokine could be expressed in the course of immune system stimulation by KLH and thus the finding would indicate an adverse effect (Aoki et al., 1995; Veselsky et al., 2003). The negative result confirms that there was no inflammatory reaction in the examined interval.

The mice receiving HI-6 only, i.e. groups 1–5, had unchanged levels of antibodies. The data from the first group which received saline only served as a control for the next experimental groups. Optical density ranged approximately from 0.17 to 0.25. Administration of KLH caused significant increase in antibody (groups 6 and 11) level compared to controls (group 1). The results from animals which received a