Effect of HI-6 on cytokines production after immunity stimulation by keyhole limpet hemocyanin in a mouse model

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Abstract

OBJECTIVES: HI-6 or asoxime in some sources is an antidotum for nerve agents. In recent experiments, implication of HI-6 in immunity response was proved; however, the issue was not studied in details. In this experiment, role of cytokines in HI-6 impact on immunity was searched.
DESIGN: BALB/c mice were exposed to saline, HI-6 in a dose 1–100 mg/kg and/or 1 keyhole limpet hemocyanin (KLH) 1 mg/kg. Mice were sacrificed 21 days after experiment beginning and interleukins (IL) 1, 2, 4, 6 were determined by Enzyme Linked Immunosorbent Assay (ELISA).
RESULTS: The animals had no pathological manifestation. From the tested cytokines, no significant alteration was found for the IL-1, IL-4 and IL-6. IL-2 was significantly increased in a dose response manner.
CONCLUSIONS: The experimental data well correlates with the previous work where HI-6 caused increase of antibodies production. HI-6 is suitable to be used as an adjuvant whenever immunity should be pharmacologically altered.

INTRODUCTION

HI-6 or asoxime is a chemical compound with proper systematic name 4-carbamoyl-1-[((2-[(E)-(hydroxyimino)methyl]pyridinium-1-yl)methoxy) methyl]pyridinium dichloride. Currently it is used as an antidotum for therapy of poisoning with some nerve agents. It causes recovery of enzyme acetylcholinesterase (AChE) after inhibiting by the nerve agents (Pohanka 2011). The HI-6 has significant ability to resolve poisoning with e.g. sarin, tabun, soman or even VX (Cetkovic *et al.* 1984; Koplovitz, and Stewart 1994). It has good efficacy toward peripheral AChE and it can partially penetrate hemato-encephalitic barrier and act central nervous system (Nyberg *et al.* 1995). Though HI-6 is known for the reactivation of AChE and recovery of its activity, it has some other target structures. However, the interaction of HI-6 with the other structures remains underestimated and not well elucidated. Especially, interaction between the oxime reactivators including HI-6 with acetylcholine receptors probably plays significant role in their action in the body (Tattersall 1993).

In recent time, effect of HI-6 on production of antibodies was revealed (Pohanka 2013). Mechanism of immunity regulation is not clear; however, regulation via cholinergic anti-inflammatory pathway was proposed as the mechanism (Pohanka 2014, 2012). The present paper is devoted to

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finding what cytokines are responsible for the effect. Knowledge about it can help to introduce HI-6 as an adjuvant to vaccines or whenever immunity should be regulated.

MATERIAL AND METHODS

Laboratory animals

BALB/c female mice were used in the experiment. The animals were received from Velaz (Unetice, Czech Republic). The animals weighted 21±3 g and were six weeks old in the experiment beginning. During the whole experiment, temperature 22±2 °C, humidity 50±10 %, and light period from 7 a.m. to 7 p.m were kept. The described experiment was approved by the Ethics Committee of the Faculty of Military Health Sciences, University of Defense (Hradec Kralove, Czech Republic).

In a total 100 mice were divided into 10 groups, each 10 animals. The animals received saline, keyhole limpet hemocyanin (KLH) 1 mg/kg in saline and or HI-6 in saline. The solutions were applied intramuscularly into rear limb in a volume 100 μl. Combination of the applied solutions is clearly visible from following items where the number of item indicates number of group: 1) controls received saline only; 2) saline and HI-6 0.1 mg/kg; 3) saline and HI-6 1 mg/kg; 4) saline and HI-6 10 mg/kg; 5) saline and HI-6 100 mg/kg; 6) saline and KLH 1 mg/kg; 7) HI-6 0.1 mg/kg and KLH 1 mg/kg; 8) HI-6 1 mg/kg and KLH 1 mg/kg; 9) HI-6 10 mg/kg and KLH 1 mg/kg; 10) HI-6 100 mg/kg and KLH 1 mg/kg.

The mice were sacrificed 21 days after tested solutions application. The interval was chosen because maximal production of antibodies can be expected in the experiment (Pohanka 2009, 2007) and the data can be compared with previous experiment (Pohanka 2013). For the sacrificing, cutting of jugular vein was chosen while the animals were in carbon dioxide narcosis.

Blood processing to plasma samples

Fresh blood was collected directly to tubes with lithium heparin (Dialab, Prague, Czech Republic). Blood was spin at 1,000 × *g* for 5 minutes. Plasma separated from sediments and stored at –80 °C until used for assay.

Enzyme linked immunosorbent assay (ELISA)

Interleukin (IL)-1β, IL-2, IL-4 and IL-6 determined in the plasma samples by ELISA. Kits RAB0274, RAB0287, RAB0299 and RAB0308 from Sigma-Aldrich (St Louis, MO, USA) were used for the purpose. The kits contained specific antibody linked to 96-well microplate and reagents for sandwich complex formation and peroxidase reaction evoking. The optical density was measured by the optical reader Sunrise (Salzburg, Austria) and concentration of the tested cytokines was calculated from calibration plots that were constructed for the purposes using attached standards.

Statistics

The experimental data were processed in software Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA). One-way ANOVA with Fisher test were made for probability levels $p<0.05$ and $p<0.01$. Standard deviation for n=10 was calculated for each group.

RESULTS AND DISCUSSION

In the experiment, no animal perished before experiment termination or exerted any pathological consequence of the exposure. The examined cytokines are presented in table 1. HI-6 alone, KLH and combination of KLH with HI-6 did not caused alteration in IL-1β, IL-4, and IL-6 plasmatic level. Comparing to the three cytokines, IL-2 was influenced by HI-6 when KLH co-applied. The effect was significant for the two upper doses of HI-6 (10 and 100 mg/kg) on probability level $p<0.01$.

The finding about IL-2 is not surprising when considered the previous work on the issue (Pohanka 2013).

Tab. 1. Summarization of cytokine levels in murine plasma.

Group	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)
1) controls	840±59	39.9±1.3	30.2±4.0	221±19
2) saline and HI-6 0.1 mg/kg	733±74	40.0±2.4	26.7±4.4	225±26
3) saline and HI-6 1 mg/kg	882±88	39.5±5.6	26.9±4.9	242±26
4) saline and HI-6 10 mg/kg	740±94	36.4±2.9	30.7±3.9	219±18
5) saline and HI-6 100 mg/kg	737±140	40.5±8.3	32.7±6.6	256±61
6) saline and KLH 1 mg/kg	873±153	39.7±5.5	28.6±6.2	249±55
7) HI-6 0.1 mg/kg and KLH 1 mg/kg	757±108	40.6±6.0	30.5±4.0	253±68
8) HI-6 1 mg/kg and KLH 1 mg/kg	705±195	43.5±4.1	30.6±6.5	221±45
9) HI-6 10 mg/kg and KLH 1 mg/kg	737±223	46.7±5.5 (**)	27.1±6.4	210±13
10) HI-6 100 mg/kg and KLH 1 mg/kg	808±178	58.7±9.7 (**)	32.3±6.6	234±47

** indicates significance against control (group 1) at probability level $p=0.01$.

The IL-2 plays an important role in T lymphocytes growth, proliferation and differentiation (Rosenberg 2014). Vaccination efficacy can be enhanced because of the IL-2 action (Karahan *et al.* 2014; Newman *et al.* 2014). No significant effect of HI-6 on IL-1β and IL-6 confirmed the fact that inflammatory reaction is not involved in the described phenomenon.

It is not easy to track pathway how HI-6 make its effect in the body. When considered the older data, HI-6 is a non-competitive inhibitor of AChE (Pohanka 2013). It has good affinity toward alpha anionic subsite of active center in the AChE that is on one hand a condition to its ability to act as oxime reactivator; on the other hand inhibitory effect in high concentration is also caused (Atanasov *et al.* 2013; Renou *et al.* 2013). Less knowledge is available about interaction of HI-6 as well as the other oxime reactivators with disparate structures including acetylcholine receptors. Though the interaction can be expected scale of the interaction and its role in the body is hardly to be inferred (Melchers *et al.* 1994; Lau 1993; Aas 1996). For the HI-6, link between immunity and cholinergic system may be mediated via cholinergic anti-inflammatory pathway where HI-6 causes higher availability of acetylcholine in the blood and the neurotransmitter then interacts with nicotinic acetylcholine receptors on macrophages (Pohanka 2014). The cholinergic anti-inflammatory pathway was proved to be effective to alter many pathological processes based on auto-immunity and/or inflammation (Tracey 2002; Rosas-Ballina & Tracey 2009; Andersson & Tracey 2012). Here, it is inferred that the proved alteration of IL-2 is caused by an indirect impact of HI-6 on macrophages via acetylcholine. Increase of IL-2 level follows and production of antibodies is enhanced as the found result.

CONCLUSIONS

HI-6 regulates immunity via IL-2. When considered the previously published data about antibodies production, HI-6 is a potent compound able modulate immunity response. This phenomenon can be used as a support when immunity response is too weak. In an example, vaccinations or anti-pathogen drugs can be enhanced by adding of HI-6 as an adjuvant. HI-6 deserves consideration in pharmacological research as a lead structure as well.

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