Immunotropic effects of undecan-2-one in mice

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Abstract

The in vivo effect of one of aliphatic 2-ketones (undecan 2-one, further named U2one) on various parameters of specific and non-specific immunity in mice were studied. The activation of respiratory burst, measured by colorimetric assay (RBA) and activation of phagocytic activity of granulocytes (PKA test), as well as increased blood and splenic lymphocytes response to LPS and ConA were observed in mice inhaled with U2one for 3 days, 5 drops for 5 mice during 1 hour daily. In mice which inhaled this for 3 days after immunisation with sheep red cells (SRBC) significant stimulation of antibody response was observed on the day 7-th. F1 hybrid mice inhaled for 3 days (days 0, + 1 and +2) after intradermal grafting of parental cells presented on the 3-rd day significantly more newly-formed blood vessels (immunological angiogenesis) than respective controls. Serum lysosyme level increased in these mice, serum levels of C-reactive protein (CRP), gamma-globulins and ceruloplasmin did not differ from the controls.

Key words: *aliphatic 2-ketones, essential oils, immunotropic activity, mice*

(Centr Eur J Immunol 2006; 31 (1-2): 57-62)

Introduction

Aliphatic 2-ketones originate as a result of a multistage metabolic transformation of fatty acids. They are present in some aromatic foods and in many essential oils of plant origin. They are also present in fruits infected with fungi [1-3]. Primate species have a well-developed olfactory sensitivity for aliphatic ketones [4]. Substantial amounts of methyl n-nonyl ketone (undecan-2-one) are present in essential oils of herbs belonging to the *Rutacea* family [5, 6], cocoanut, peanut, cottonseed, sunflower seed oils [1] and some amounts are present also in fruits, flowers, and cortex of other plants (*Rubus idaeus L.*, *Pistacia Lentiscus L.*, *Glycosmis pentaphylla*, *Cambopogon schoenanthus*, *Cinnamonium glaucescens*, and in essential oils of ginger, chinese *Rosa rugosa* and turkish *Salvia blepharochlaena* [7-12]. Methyl ketones are also present in odorous secretions of some insects [1] where they function as defensive, offensive, alerting and alarm substances. Aliphatic undecanones are often used in food and fragrant industries, owing to their nice flavours.

Aromatherapy has been practiced for ages. Botanical perfumes, incenses and ointments were used in ancient China, India and Egypt. Aromatic substances of plant origin were used in religious ceremonies, for culinary purposes, as cosmetics, and as strong anti-septic substances. Egiptian, Greek and Roman physicians recommended fragrant oils for treatment of various diseases (by bathing, massage or aromatherapy) and also for other purposes (embalming) [13].

There is a long history of therapeutic uses of some essential oils – among them lavender oil and tea-tree (*Melaleuca alternifolia*) oil, recognized as strong antimicrobial factors [14].

The high effectiveness and wide range of applications of these oils suggest, that besides their antiseptic activity, these substances may have some immunomodulatory properties. In fact, our previous studies for the first time have revealed their strong immunostimulatory properties [15-18]. However, we observed the opposite effect of some other aromatic oils (unpublished) – some of them (synthetic almond oil, manuka essential oil) suppressed antibody production in inhaled mice, which is presented on Fig. 1.

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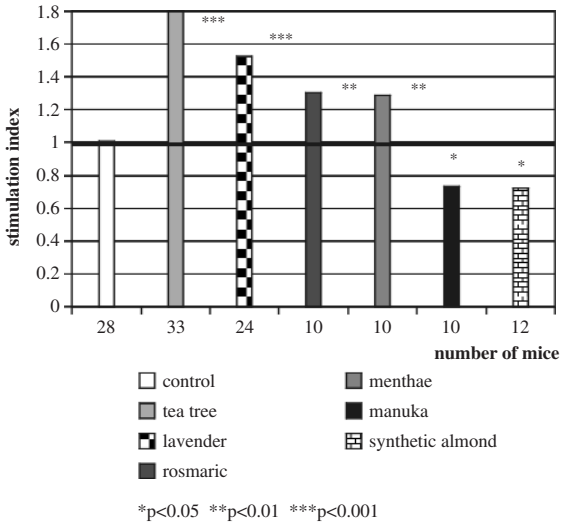

Fig. 1. The comparison of the effect of various essential oils on antibody production. Mice were inhaling oils for 5 days AFTER contact with antigen (SRBC)

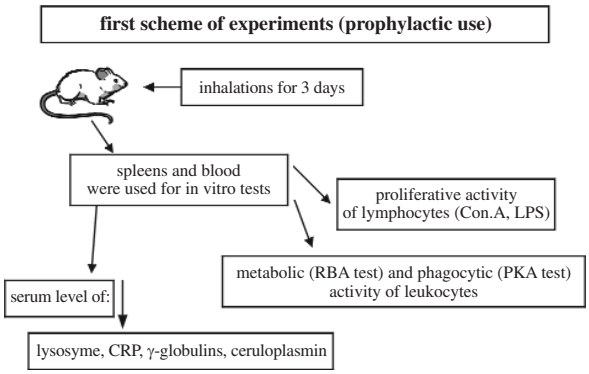

Fig. 2. First scheme of experiments (prophylactic use)

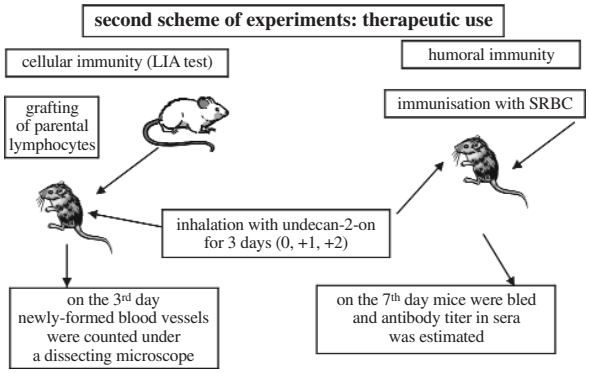

Fig. 3. Second scheme of experiments: therapeutic use

It was the reason to begin the present study on the influence (positive or negative) of some flavours (used in food and cosmetic industries) on cellular and humoral immune response. Since there have been no reports so far on the immunotropic activity of undecan-2-one in vivo, we decided to investigate the effect of this compound on various parameters of specific and non-specific humoral and cellular immunity in mice.

Materials and methods

Chemical

Undecan-2-one (U2one) was prepared by ketonization of a mixture of acetic and decanoic acids in the presence of 20wt% MnO_2/Al_2O_3 catalyst under flowing conditions. Yield of ketone 70%, purity 99,9% (GC), n^{20}_D=1,4262 (exp). The comprehensive description of its synthesis, the physical and spectral properties were given elsewhere [19]. The obtained undecan-2-one has a fruity-floral odour with an orange-herbaceous note [20].

Experimental design

The study was performed on 10-12 week old inbred Balb/c mice, weighing 25-28 g, of both sexes, delivered from a breeding colony of the Polish Academy of Sciences, and Warsaw and Mazurian (Olsztyn) Universities, and on F1 hybrids (Balb/cxC3H and Balb/c x DBA2), from their own breeding colony.

Mice were subjected to inhalation for 3 days, according to the following scheme: 5 mice in one cage, 5 drops of U2one for 60 minutes, cage covered by linen during inhalation. Cages with control mice were accordingly covered by linen for 60 minutes [18].

A part of the mice was subjected to inhalations before taking material (blood and spleen) for examination of cellular and humoral immunity (prophylactic scheme 1, Fig. 2), a part was subjected to inhalations after contact with sheep red blood cells, SRBC, or allogeneic histocompatibility antigens (therapeutic scheme 2, Fig. 3).

Methods (prophylactic scheme 1)

Mice were anaesthetized with chloral hydrate, bled from retroorbital plexus and sacrificed with Morbital. Splenocytes were isolated from mice under sterile conditions by straining spleens through stainless sieve and cotton gauze and centrifugation on Gradisol (Aqua -Medica, Poland) in order to remove erythrocytes.

Leucocytes were isolated from blood by centrifugation at 2000 g for 30 min at 4°C on the Gradisol L or G gradient (Aqua-Medica, Poland), washed three time in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2 x 10^6 cells/ml of medium. Viability of cells was checked by supravital staining with 0.1 % w/v trypan blue [21].

In vitro *ex vivo* study of cell-mediated immunity

1. RBA test. The metabolic activity of blood phagocyting cells (mostly granulocytes) was determined based on the measurement of intracellular respiratory burst after stimulation by PMA (phorbol myristate acetate, Sigma), as described by Chung and Secombes [21] and adapted for dogs by Siwicki et al. [22]. The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10^6 cells/ml. On 96-well U-shaped microplates 100 µl of isolated blood leukocytes were mixed with 100 µl of 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2 and 1 µl of PMA at concentration 1 mg/ml in ethanol was added. After 30 min of incubation at 37°C, the supernatant was removed from each well. The cells pellet was washed with absolute ethanol and then three times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT after incubation with 2 M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value given as the result.
2. PKA test. Potential bactericidal activity of phagocyting cells was determined in isolated blood leukocytes stimulated with killed microorganisms, according to the method presented by Rook et al. [23]. On 96-well U-shaped microplates 100 µl of leucocytes were mixed with 100 µl of 0.2% NBT in phosphate buffer at pH 7.2 and 10 µl of killed *Staphylococcus aureus* strain 209P (containing 10^6 bacteria) were added. The mixture was incubated 1h at 37°C and the supernatant was removed. The cell pellet was washed with absolute ethanol and then three times with 70% ethanol and dried at room temperature. This was followed by the addition of 2M KOH and DMSO to each well. The amount of extracted reduced NBT was measured at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.
3. The proliferative response of splenic and blood lymphocytes. A proliferative responses of lymphocytes, stimulated by mitogen concanavaline A (ConA) or lipopolysaccharide (LPS), were determined by MTT assay [24, 25]. MTT [3-(4.5-Dimethyl thiazol-2-yl) 2.5-diphenyl-tetrazolium bromide] (Sigma) was dissolved in PBS at concentration of 5 mg/ml and filtered. On 96-well culture plates (Costar, USA) 100 µl of blood lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100U/100 µcg/ml) were mixed with 100 µl of RPMI 1640 containing mitogens ConA (5 µcg/ml), PHA (10 µcg/ml) or LPS (20 µcg/ml). Three cultures from each pool of leukocytes were established. After 72 h of incubation at 37°C in the atmosphere of 5% carbon dioxide atmosphere (Assab Incubator, Sweden), 50 µl of MTT solution were added into each well and plates were incubated for 4h at 37°C. After the incubation the plates were centrifuged (1400 g, 15°C, 5 min). Supernatants were removed and 100 µl of DMSO (Sigma) were added into each well and incubated for 15 min at room temperature. After the incubation the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech).

The results from three cultures were collected. The mean values and standard errors from them were used for comparison between the groups by the Student's *t* test.

Lysozyme, gamma-globulins, C-reactive protein (CRP) and ceruloplasmin levels were determined according to the methods described [26].

Methods – „therapeutic" scheme 2

Study of antibodies production [27]. F1 (DBA2xBalb/c) 12 weeks old female mice were immunized with 10% SRBC (0,1 ml intraperitoneally), subjected to inhalation in days 0, +1 and +2 and were bled 7 days after immunization. Antibody level was evaluated with haemagglutination assay in inactivated (56°C, 30 min) sera. After performing serial sera dilutions, 0.5% SRBC were added and the mixture was incubated for 60 min at room temperature, then centrifuged (10', 150 g) and shaken. Hemagglutination titer was evaluated in light microscope – as the last dilution where at least 3 cells conglomerates were present in at least 3 consecutive fields at objective magnification 20x.

Local graft-versus-host reaction (immunological angiogenesis,LIA test):

A local GvH reaction (lymphocyte- induced angiogenesis test, LIA) was performed according to Sidky & Auerbach [28] with some modifications [27]. In short, 12 weeks old female Balb/c mice were sacrificed with Morbital, spleens were dissected and spleen cells suspensions were grafted intradermally (500 thousands of cells in 0.05 ml of Parker medium per graft) into 12 weeks old (Balb/c x C3H) F1 mice, female. Before performing injections mice were anaesthetized with 3.6% chloralhydrate (0.1 ml per 10 g of body weight). Both flanks of each mouse were finely shaved with a razor blade, each flank was injected 2-3 times. Cell suspensions were supplemented with 0.05 ml/ml of 0.01% trypan blue in order to facilitate recognition of injection sites later on.

Grafted Balb/c spleen cells recognized C3H antigens and produced many immunological mediators including proangiogenic factors (immunological angiogenesis). In this case the number of newly formed blood vessels was the measure of cell reactivity. After 72 hours the mice were treated with a lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope on the inner skin surface, using criteria suggested by the authors of the method, at magnification of 6x, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cell injection, differ

Table 1. The effect of 10% solution of undecan-2-one on cellular immunity ("prophylactic" scheme)

Test	Mean OD 620 nm±SE			
	Blood		Spleen	
	Control (n=5)	U2one (n=5)	Control (n=5)	U2one (n=5)
RBA	0.32±0.018	0.40±0.013*	not tested	not tested
PKA	0.30±0.013	0.39±0.009*	not tested	not tested
ConA	0.44±0.009	0.50±0.013*	0.45±0.018	0.56±0.018*
LPS	0.35±0.013	0.43±0.008*	0.38±0.018	0.45±0.013*

** p<0.05*

Table 2. The effect of 10% solution of undecan-2-one on some humoral factors level in sera ("prophylactic" scheme)

	Control mice (n=5)	U2one inhaling mice (n=5)
Lysozyme (mg/l±SE)	7.85± 0.2	8.65±0.16*
CRP (mg/l±SE)	25.5±1.14	26.5±0.92 n.s.
Ceruloplasmin (IU)±SE	19.5±0.54	18.7±0.67 n.s.
γ-globulins (g/l±SE)	9.25±0.54	10.1±0.55 n.s.

** p<0.05 n.s.: difference nonsignificant*

from the background vasculature in their tortuosity and divarications.

All experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass), and were approved and supervised by Local Ethical Committee. Statistical analysis was performed by Mann-Whitney and Student *t* tests.

Results

Inhalation of mice with 10% undecan-2-one for 3 consecutive days enhanced their immune response to antigens and mitogens. We observed an increased metabolic and phagocytic activity of blood leukocytes, and an increased proliferative response to mitogens of blood and splenic T (Con A) and B (LPS) lymphocytes (Table 1). The lysozyme level in sera of inhaled mice was higher than in the respective controls.The acute phase proteins (CRP, ceruloplasmin) level did not differ from the control, non-inhaling group, similarily as the gamma-globulin concentration in sera (Table 2). Inhalation of mice after their contact with antigen („therapeutic" scheme) resulted in a significantly higher neovascular reaction in the local graft-versus-host test (LIA) (Fig. 4) and an increased antibody production to SRBC in comparison to the controls (Table 3). Higher (50% and 100%) concentrations of U2one increased antibody production also, but the best effect was obtained for 10% solution of this substance (p<0.01).

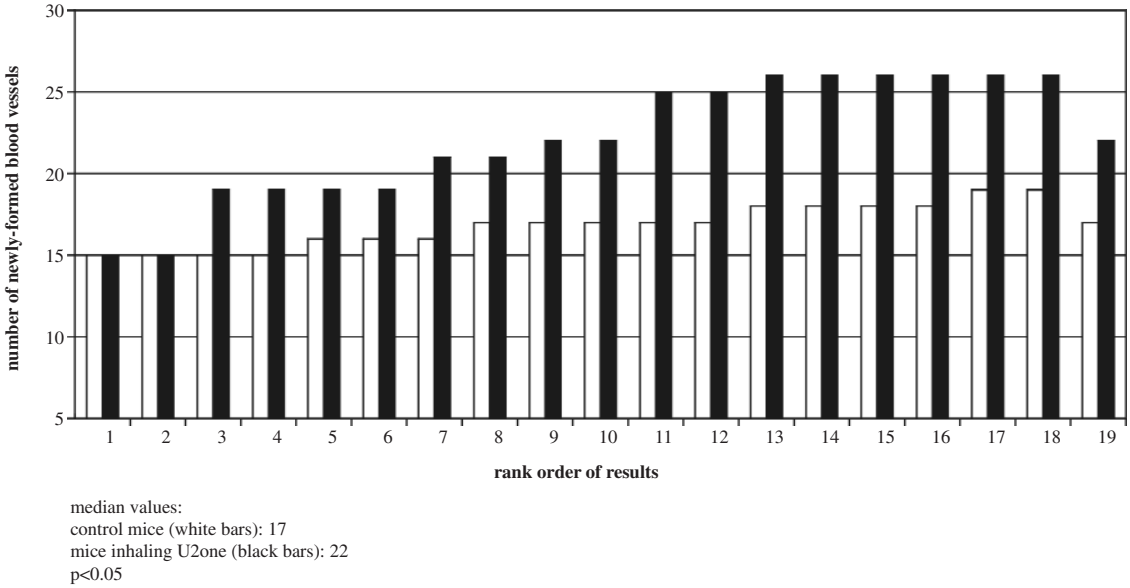

Fig. 4. The effect of inhalations on neovascular response induced in F1 mice by parental lymphocytes

Discussion

In this paper we show for the first time immunostimulatory properties of methyl n-nonyl ketone (undecan-2-one), a substance present in various plants and foods. Mice subjected to inhalations with this substance for 3 days presented an enhancement of cellular and humoral immunity disregarding whether inhalations were performed before or after contact with antigens or mitogens. Up to date, U2one has been recognized as an anti-fungal, anti-bacterial, anti-viral and a cytotoxic substance. There is a report on the lack of its influence on the growth of *Raphanus sativus L.* seeds in in vitro culture [29]. Other authors reported some toxicity to viruses. Antiviral activity of steam distillate from *Houttuynia cordata* and one of its components, U2one, was observed against HSV-1, against influenza virus, and against HIV-1. No loss of the anti-viral activity was observed after 6 months of storage at 4°C [30]. The authors have suggested interference with virus envelope, as possible mechanism of this reaction, which results in the loss of infectivity. Anti-viral concentrations were much less than concentrations toxic ones for human epithelioid cervical carcinoma, African green monkey kidney and canine kidney cell lines.

Undecan 2- one, together with other 2-ketones is formed during the frying of pork meat in butter, frying pork in sunlower oil results in a high aldehyde aliphatic content, and volatiles detected in pig-lard fried pork presented some other aldehydes [31]. In our opinion, the knowledge of biological functions of all these volatile products of frying, among them, their possible influence on immunity, is very important to establish correct dietetic rules.

A fragrance stimulates the olfactory system, both in primates [4] and in rodents [32]. During inhalation, chemical compounds travel to the olfactory bulb and then to the limbic part of the brain. The amygdala governs our emotional response, hippocampus the memory and recognition of smell [33]. Might be the brain indirectly involved in immuno-enhancing effects observed by us in mice inhaling undecan-2-one? It is known, that endorphins may stimulate immunological responses, but there are no available data about the possible effect of undecan-2-one or other 2-ketones on endorphin production. The mechanism of influence of those compounds on neuroimmunological processes remains unclear. This can be associated possibly with the function of cholinergic and adrenergic nerves as well as NANC-type (*noncholinergic, nonadrenergic*) nerves determining secretion of neurotransmitters, e.g. catecholamines, endorphins, met-enkephalins, NO and other substances. Changes of the levels of those factors can lead to the modulation of both specific and non-specific immune responses [34-36]. It should be emphasised that the olfactory system comes into contact with the above mentioned neurotransmitting routes [37, 38].

At present, it cannot be also excluded that the studied compounds can influence the non-neuronal cholinergic system in lymphocytes [39], affect the expression of β_2-adrenergic receptors on T $CD4^+$ cells and B-cells and norepinephrine secretion [40].

Table 3. The effect of undecan-2-one on antibody production in mice ("therapeutic" scheme)

Tested material	Number of mice	Mean log of titer±SE	Difference from the control
control	10	7.7±0.14	-
U2one 10%	5	10.6±0.15	p<0.01
U2one 50%	5	8.6±0.15	p<0.05
U2one 100%	5	8.6±0.15	p<0.05

Studies of the effect of *Radix Angelicae Dahuricae* essential oil, performed on pain model in rats showed that this substance caused a significant increase of the levels of β-endorphin and NO and an increase of expression of mRNA for propiomelanocortin in the hypothalamus and brainstem [41].

Owing to the lack of papers about the possible mechanism of action of 2-ketones on mammalian granulocytes and lymphocytes, we cannot formulate a hypothesis explaining the U2one immunostimulatory effect. However, the problem is very important and we will continue our studies on this topic.

Acknowledgments

This study was supported by grant KBN 3 T09B 062 28

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