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[1*]R.T. Kenzhebekova, [2]A.O. Abekova, [1]K.D. Raziyeva,
[1]Zh.S. Abramova, [1]R.A. Islamov, [3]A.K. Nersesyan, [1]A.I. Ilin

[1]JSC «Scientific Center for Anti-Infectious Drugs», Almaty, Kazakhstan
[2]al-Farabi Kazakh National University, Almaty, Kazakhstan
[3]Institute of Cancer Research, Medical University of Vienna, Austria
[*]e-mail: nurrosenur@inbox.ru

Investigation of the impact of iodine coordination compound on production of interleukin-4 and interferon-γ *in vitro* and primary evaluation of local irritation *in vivo*

Abstract: It is known that some iodine-containing drugs have pleiotropic effects on the body's immune response. The immune system can both inhibit and activate the production of pro-inflammatory and anti-inflammatory cytokines in response to stimuli. Those properties can be valuable when developing drugs for various purposes as well as for evaluating their safety. Key regulatory cytokines that can be involved in the response to iodine are IL-4 and IFN-γ. Therefore, relation of those cytokines is often regarded as a marker when evaluating cellular and humoral immunity, including allergies. Studies of an allergic response to iodine give conflicting results. Difficulties in interpreting results are usually associated with the fact that molecular iodine is not able to cause an allergic reaction on its own. However, by acting as a hapten, iodine is capable of forming bounds with the body's proteins and consequently can induce immune response to iodinated proteins. Thus, by studying production of IL-4 and IFN-γ we can characterize effects of new iodine coordination compound on some components of the immunity. This paper presents the results of our study, where we evaluated cytotoxicity and the ability to induce production of IL-4 and IFN-γ cytokines by MDCK (Madin-Darby canine kidney cells) and PBMN (mononuclear cells from peripheral blood) after treatment with a new coordinated compound (KC). KC is a drug that contains a multiple polymer complex comprising of molecular iodine coordinated by lithium and potassium halides, di- and tri-peptides and α-dextrin. An induction of IFN-γ was only detected in PBMN cells treated at concentrations close to 50% cytotoxic concentration (CC_{50}), which can be interpreted as a cytotoxic effect. *In vivo* study showed the absence of irritating effect on the skin of tested animals. Treatment had no impact on the body weight of rabbits. Results of this study add new knowledge to the influence of iodine complexes on immunity.
Key words: iodine coordination compound, molecular iodine, toxicity, cytokine, local irritation effect.

Introduction

Iodine is a micronutrient that is vital at all stages of life and is of a crucial importance for the proper function of all organ systems [1]. Iodine complex compounds are applied to a broad spectrum of procedures in medicine in form of biocides [2]. Molecular iodine and its complexes also show properties of antineoplastic agents, by inhibiting proliferation of cancer cells [3]. However, practical use of iodine complexes shows negative side effects in some cases. Some of the published work describes various harmful effects of iodine, such as thyroid toxicity, local irritating effect on mucosa and so-called iodine allergy [4-6].

In clinical practice, the term "allergy" is used to refer to a broad variety of immune responses. Iodine does not have antigenic properties, which means that it is not recognized by the immune system and is not able to trigger an allergic reaction by itself. However, iodine may cause immune responses as a hapten molecule when attached to larger proteins [7; 8]. Povidone iodine, which is widely used in medicine, may cause adverse skin reactions from a simple skin irritation to a serious rash resembling chemical burn. Iodinated contrast dye, which is administered intravenously as an X-ray radiocontrast agent can cause an allergic reaction, in some cases leading to anaphylaxis [9-11].

In addition, iodine solutions produce diverse effects on the production of certain cytokines. An in-

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duction of TNF-α is effectively inhibited by iodine solution in case of rabbit skin inflammation after treatment with mustard gas [12]. Similar effect is observed when iodine solutions are administered to rats with testosterone-induced oxidative stress [13].

Contrariwise, an excess of iodine intake in form of iodides leads to increased level of IL-6 and TNF-α in serum [14]. Treatment of lymphocytes with Lugol's solution *in vitro* results in the production of TNF-α, but not IL-6 or IL-8 cytokines. Simultaneously, interaction of KI_3 with keratinocytes does not induce cytokine response [15]. An oral rinse with iodine solutions in chronic gingivitis reduces clinical manifestations of inflammation as well as the level of IL-2 and IFN-γ production in biopsy material [16]. It is well known that a high level of iodine intake can activate autoimmune mechanisms [17]. This pathological process underlies the autoimmune diseases of the thyroid gland [18]. It should be noted that an effect is dependent on the chemical form of iodine entering the body.

Thus, for the proper evaluation of immune responses 2 types of cytokines have to be studied, which are proinflammatory (type 1 cytokines, including interleukin-2 (IL-2) and interferon-γ (IFNγ), that promote cell-mediated immune responses; and anti-inflammatory (type 2 cytokines, including IL-4 and IL-10), that promote antibody production and allergy.

Therefore, the purpose of this work was to study an effect of a new iodine coordinating compound (KC) on the production of IL-4 and IFN-γ. Cell lines that were used as models are human peripheral blood mononuclear cells (PBMN) and Madin-Darby canine kidney cells (MDCK) that are able to form multilayer culture and produce some cytokines [19]. Rabbits were used as *in vivo* models for primary evaluation of local irritation.

Materials and methods

Test substance. KC is a multiple polymer complex consisting of molecular iodine coordinated by lithium and potassium halides, di- and tri-peptides and α-dextrin [20].

Cells. MDCK cell line was purchased from Russian Cell Biotechnology Laboratory and PBMN cells - from RSE on REU "Republican Blood Center" of the Ministry of Healthcare of the Republic of Kazakhstan, donor denotation code 1410104939, contract No. 49, from March 1, 2018.

Isolation of mononuclear cell fraction. Human peripheral blood was pre-mixed with 6% dextran solution in a 50 mL polypropylene centrifuge tube to precipitate erythrocytes. Then, the supernatant was washed, resuspended and fractionated by density gradient centrifugation on Ficoll-Paque (Sigma, USA), corresponding to the floating density of human mononuclear cells (density = 1.078) at 4 ºC, 3000 rpm for 20 minutes (Centrifuge 5810R; Eppendorf, Germany). The mononuclear cell fraction was washed by centrifugation and resuspended in RPMI-1640 culture medium (Sigma, USA). The percentage of viable cells was assessed using trypan blue incorporation. In all the experiments, suspensions with a percentage of viable cells greater than 90 % were used.

Cell culturing. Cells were cultured in RPMI-1640 medium supplemented with 10 % heat inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ºC in humidified environment of 5 % CO_2 for 24 hours before treatment. Cell viability was assessed by trypan blue (Sigma, USA). Cell culture with the percentage of viable cells greater than 90 % was used in the experiment.

Cytotoxic effects of KC. The cytotoxicity of KC determined by cell uptake of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, USA) colorimetric assay. Estimation of cell viability by MTT method is based on the measurement of cellular mitochondrial dehydrogenase activity [21]. Cells were cultured in 96-well plates (BRAND plates, Germany) at a concentration of $3x10^4$ cells/well, including 200 μl fresh medium. After 24 hours of incubation, growth medium was removed from wells and 200 μl of KC diluent containing seven different concentrations, namely: 0.01, 0.06, 0.31, 0.78, 1.56, 3.91 and 7.82 mM, were added. Wells with negative control samples contained 200 μl of nutrient medium without any substance. Photometric measurement of the optical density of dissolved formazan was performed on a Sunrise RC.4 microplate reader (Tecan, Austria) at the wavelength of the main filter of 540 nm and a reference wave of 620 nm. To calculate 50 % cytotoxic concentration (CC_{50}) GraphPad Prism V6 (GraphPad Software; La Jolla California USA) was used for each tested compound.

Study of IFN-γ and IL-4 cytokines production after KC treatment. IFN-γ was evaluated by enzyme-linked immunosorbent assay using a commercial kit Gamma-Interferon (JSC Vector-Best, Russia). IL-4 was evaluated by enzyme-linked immunosorbent assay using a commercial kit IL-4 (R&D Systems, USA). To determine the production of IFN-γ and IL-4, mononuclear and epithelial cells suspensions were dispersed into 96-well plates (BD Falcon, USA) at a concentration of 10^5 cells/well and incubated in a CO_2 incubator for 24 hours in complete culture me-

dium containing various concentrations of KC. 10% RPMI-1640 culture medium was used. Sterile deionized water was used as a negative control in all experiments. ConA was used as a positive control. It is widely used as a potential agent able to induce production of a wide range of cytokines *in vitro* [22]. For ELISA assay cell culture supernatant was used. Measurement of optical density was performed on a Sunrise RC.4 microplate reader (Tecan, Austria) using Magelan 2.0 software (Tecan, Austria) at a wavelength of 450 nm with a reference filter at 650 nm. Concentration of released cytokines and standards was calculated using GraphPad Prism V6 (GraphPad Software; La Jolla California, USA).

Skin irritation test on animals. Three male rabbits were obtained from the Scientific and Practical Center of Sanitary and Epidemiological Expertise and Monitoring (Almaty, Kazakhstan). The animal studies were conducted in accordance with the procedures and principles outlined in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH, USA). The rabbits were individually housed in environmentally monitored and ventilated rabbit experimental room I, maintained at 15-21°C temperature and a relative air humidity of 30-70%. Feed and water containers were changed and sanitized once a week at least. Before the study, rabbits were acclimated for 24 days. The study was approved by the Ethics Committee of the Scientific Center (Protocol No. 25/13).

The study was started with administration of one animal. No signs of irritation were observed within 72 hours, thus next two animals were administered in the same manner. Approximately 24 hours before the study, fur was removed by closely clipping the dorsal area of animal trunks, approximately 10 x 15 cm in size. The operation was performed carefully to prevent abrading of the skin, which could alter its permeability. Only animals with healthy intact skin were used. The KC in the amount of 0.5 ml was administered on application site of 2.5 x 2.5 cm in size. After the administration the corresponding skin surface was covered with a patch and non-irritating tape. Control sites were administered with 0.5 ml of water for injection only and the corresponding skin surface was covered with non-irritating patch and tape (2.5 x 2.5 cm in size). The exposure period was 4 hours. At the end of the exposure period residual application form was removed with a small quantity of water for injection. All the rabbits were individually weighed at delivery before administration and at the end of the study. The animals were euthanized by injecting the overdose of pentobarbital at the end of the study.

Statistical data analysis. Results are presented as the means ± SD of three independent experiments. The method of dispersion analysis with nonparametric procedures was used in this study according to the abnormal distribution of data. Statistical significance of experimental values when comparing with control was assessed by the GraphPad Prism version 6 for Windows (GraphPad Software; La Jolla California, USA), using Mann-Whitney test, Kruskal-Wallis one-way analysis and nonlinear standard curves for ELISA test. Values of $P>0.05$ were considered as insignificant.

Results and discussion

Cytotoxicity of KC. CC_{50} was calculated after treatment of MDCK and PBMN cells with KC using Hill equation in GraphPad Prism (Table 1). The difference between concentrations of two cell lines was non-significant ($P>0.05$). No selectivity was found. The results also indicate that iodine had a direct effect as an oxidizing agent on membranes of MDCK and PBMN cells.

Table 1 – Comparative CC_{50} values of KC in monolayer and suspension cells

Cell lines	CC_{50} (Mean ± SD), mM
MDCK	1.45 ± 0.22
PBMN	0.74 ± 0.04

In dose-response analysis for KC on a logarithmic scale after 24 hours of exposure (Figure 1) KC "dose – response" log-curves were characterized by a classical sigmoidal shape.

According to the chart, MDCK line is steeper than that of PBMN. This can be explained by the difference in the type of cultured cells, monolayer and suspension.

The level of the production of IFN-γ and IL-4 cytokines. ELISA assay was done to determine the levels of cytokines in MDCK and PBMN cell supernatants pre-treated with KC. MDCK culture did not produce IFN-γ and IL-4 after 24 hours of exposure with KC (Table 2).

However, KC induced production of IFN-γ by human mononuclear cells at high concentrations (Table 3).

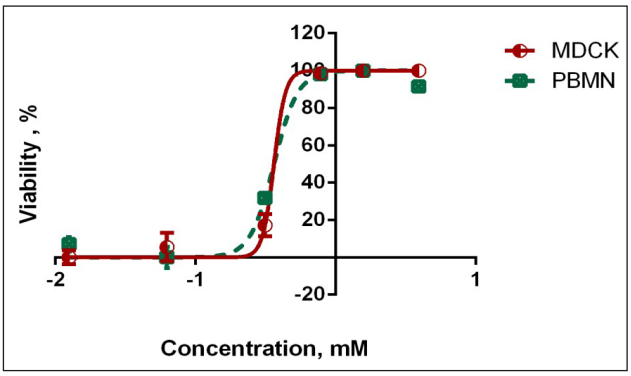

Figure 1 – Log-curves "dose – response" for KC
Note: MDCK – MDCK cell,
PBMN – mononuclear cells from peripheral blood

Table 2 – Production of cytokines by MDCK after 24 hours of treatment

KC concentrations, mM	Cytokine concentrations, pg/ml	
	IFN-γ	IL-4
NC	1.56 ± 0.67	1.76 ± 0.71
PC	13.71 ± 1.05*	29.05 ± 1.10*
7.82	1.15 ± 0.06	1.12 ± 0.32
3.91	1.78 ± 0.13	2.00 ± 0.67
1.56	0.00	1.50 ± 0.56
0.78	1.77 ± 0.51	0.00
0.31	0.00	1.48 ± 0.54
0.06	1.82 ± 0.54	1.82 ± 0.80
0.01	0.00	1.28 ± 1.47
Note: NC – negative control, PC – positive control; * P<0.05 vs NC		

Iodine complexes and solutions (KI_3 and PVP-iodine), when exposing locally to both damaged and healthy epithelial tissue *in vivo* or *in vitro* can either suppress the production of TNF-α and IL-2 proinflammatory cytokines or not act at all [23-25]. Due to such features it is often concluded that iodine solutions have anti-inflammatory properties [26]. However, we observed that an effect on cytokines production depends on the type of cells used. For example, treatment with high concentrations of KC showed that only PBMN cells are able to response with the production of solely IFN-γ cytokines. We did not observe a response from MDCK cells. It should be noted that the studied cytokine IL-4 plays an essential role by promoting Th2 cell differentiation while inhibiting Th1 cell differentiation. The pleiotropic effect of IL-4 reveals the important role that this cytokine plays during a normal immune response [27]. Presence of IL-4 during *in vitro* priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. An association of IL-4 with allergic reactions is also significant. IL-4 increases production of IgE [28]. Nevertheless, the lack of IL-4 production and increase of IFN-γ expression when administering KC at high-doses (3.9–7.8 mM) can only be interpreted as cytotoxic effect of KC on PBMN cells. Correlation of IL-4 and IFN-γ and their imbalance are often considered as indicators of certain pathological processes. Therefore, the absence of PBMN cells reaction to KC at concentrations lower than 1.5 mM can indicate lack of disregulating properties on the studied components of the immune system [29-31].

Table 3 – Production of cytokines by PBMN after 24 hours of treatment

KC concentrations, mM	Cytokine concentrations, pg/ml	
	IFN-γ	IL-4
NC	1.56 ± 0.67	0.00
PC	39.13 ± 1.05*	6.07 ± 1.31*
7.82	791.02 ± 1.56*	0.00
3.91	900.73 ± 0.95*	0.00
1.56	0.00	0.00
0.78	0.00	0.00
0.31	0.00	0.00
0.06	0.00	0.00
0.01	0.00	0.00
Note: NC – negative control, PC – positive control; * P<0.05 vs NC		

Skin irritation test on rabbit. All rabbits were observed daily for clinical signs, morbidity or mortality during the study. Changes in the skin, gait, somatic activity and behavior pattern were also observed. The visual aspect of each application site was evaluated in 1 hour after treatment. After removing the patch, time intervals of 24, 48, and 72 hours were investigated and the skin reaction for erythema and oedema was graded according to the following table classification system for skin reaction (ISO 10993-10). The

daily clinical observation of the general health status of all tested animals revealed no pathological abnormalities.

The erythema and oedema on rabbits were not observed. The irritation index was 0. Therefore the irritation response has been defined as negligible. The body weight (Table 4) of the animals was not negatively affected by the KC application. The body weight increases of all the rabbits were normal and corresponded with their age.

Table 4 – Body weight of rabbits, kg

Animal No.	At delivery	Before administration	48 hours after administration	72 hours after administration
M 1	2.90	3.18	-	3.38
M 2	3.00	-	3.38	3.58
M 3	2.68	-	3.20	3.29

Evaluation of local irritating effect of iodine-containing drugs (primarily PVP-iodine) remains relevant since there are clinical incidences of contact dermatitis in some patients in the postoperative period [32]. Moreover, a degree of irritation with PVP-iodine is dose-dependent [32; 33]. Despite the development of alternative test methods *in vitro*, clinical manifestation of the irritation effect to test substance is a valuable prognostic marker [34]. The results, obtained from evaluating KC irritating effect on rabbits' skin, showed the absence of damaging properties. Some researchers believe that the complexation of iodine with polymers can reduce the irritating effect of iodine [35]. Molecular iodine, which is contained in KC, is localized inside of the dextrin helix in form of a polyiodide. Peptides create a competitive environment with body proteins, which ensures high stability of the complex [36]. These features may possibly explain the absence of disregulating properties on some components of immunity and local irritation.

Conclusion

Madin-Darby canine kidney cells (MDCK) and mononuclear cells from peripheral blood (PBMN) were treated with a new iodine coordination compound (KC). Close to 50% cytotoxic concentration (CC_{50}) is estimated as 1.45 mM for MDCK and 0.74 mM for PBMN. We detected an induction of IFN-γ in PBMN cells treated with KC at concentrations close to CC_{50} (from 7.82 to 3.91 mM), which can be interpreted as a cytotoxic effect. KC also does not irritate rabbits' skin. The results, obtained from evaluation of KC irritating effect on rabbits' skin, showed the absence of deteriorative properties. Results of this study add new knowledge to the influence of iodine complexes on immunity. Obviously, future experiments aim to analyze immune response, through other key cytokines production.

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