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Original Research

Evaluation of Some Immunoregulatory Cytokines in Serum of Rats Exposed to Cadmium and Ethanol

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Abstract

The aim of the present study was to investigate the effects of cadmium (Cd) (5 and 50 mg Cd/l in drinking water) and ethyl alcohol (ethanol, EtOH) (5 g EtOH/kg b.wt., intragastrically), administered alone or simultaneously, on the concentrations of pro-inflammatory (interleukin-1, IL-1α; interleukin-6, IL-6; tumor necrosis factor-α, TNFα and interferon γ, INFγ) and anti-inflammatory (interleukin-4, IL-4) cytokines in the serum of rats. In order to estimate the involvement of Cd- or/and EtOH-induced oxidative stress in damage to cytokines, the concentration of protein carbonyl groups (PC), as a marker of oxidative protein damage, was also determined.

Exposure to 5 and 50 mg Cd/l, alone or in combination with EtOH, led to an increase in the serum concentrations of IL-1α, TNFα and INFγ with a simultaneous decrease in IL-4 concentration, compared to the control. The concentration of IL-6 was elevated only after administration of 50 mg Cd/l, both alone and in combination with EtOH. The exposure to EtOH alone resulted in increased concentrations of TNFα and INFγ, as well as in decreased concentrations of IL-4. In rats co-exposed to Cd and EtOH, the changes observed in the concentrations of the cytokines, except in IL-6, were more advanced, compared to the animals treated with these xenobiotics alone. Exposure to Cd and EtOH, both alone and in combination, caused an increase in the serum PC concentration. The concentration of PC positively correlated with the concentrations of IL-1α, IL-6, TNFα and INFγ and negatively with IL-4 concentration.

The results suggest that changes in the cytokines examined are more enhanced after combined exposure to Cd and EtOH, especially at higher Cd dosage. Moreover, it can be hypothesized that oxidative stress may be involved in the mechanism leading to changes in the concentration of cytokines after exposure to Cd and EtOH alone and in combination.

Keywords: cadmium, ethanol, cytokines, protein carbonyl groups, rat

Introduction

Cadmium (Cd) is one of the main environmental and occupational pollutants in industrialized countries that pose a serious health threat [1-4]. Excessive ethanol (EtOH) consumption is another health-threatening problem [5, 6]. Because ethanol (EtOH) abusers can also be occupationally or environmentally exposed to Cd, interactions between these two substances are an important epidemiological and medical issue.

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Chronic exposure to Cd leads to damage to numerous organs and systems, primarily the kidneys and skeleton [1, 2, 7-9]. Long-term EtOH consumption damages mainly the liver [9, 10]. Recently, the immunomodulatory effects of Cd and EtOH have also been demonstrated. Cd induces disorders in the humoral and cellular immune responses, affects B and T lymphocytes, macrophages and such cytotoxic cells as K (killers) and NK (natural killers) cells [11-15]. EtOH affects both innate and adaptive immunity [16]. EtOH can impair normal functioning of neutrophils, macrophages and NK cells [16, 17], and influence the number and function of T and B cells [18, 19].

Immune cells produce mediators of inflammatory and immune reactions called cytokines. These low-molecular-weight glycoproteins in small concentrations are indispensable for normal functioning of the immune system. Their excessive secretion, however, leads to immune cell dysfunctions. Inflammatory cytokines may be produced by mononuclear cells of the immune system in response to numerous agents, such as microorganisms and these products (e.g., lipopolysaccharide – LPS) [20, 21], as well as some xenobiotics [22]. Both Cd [11, 15, 23, 24] and EtOH [6, 25, 26] may affect secretion of inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interferon γ

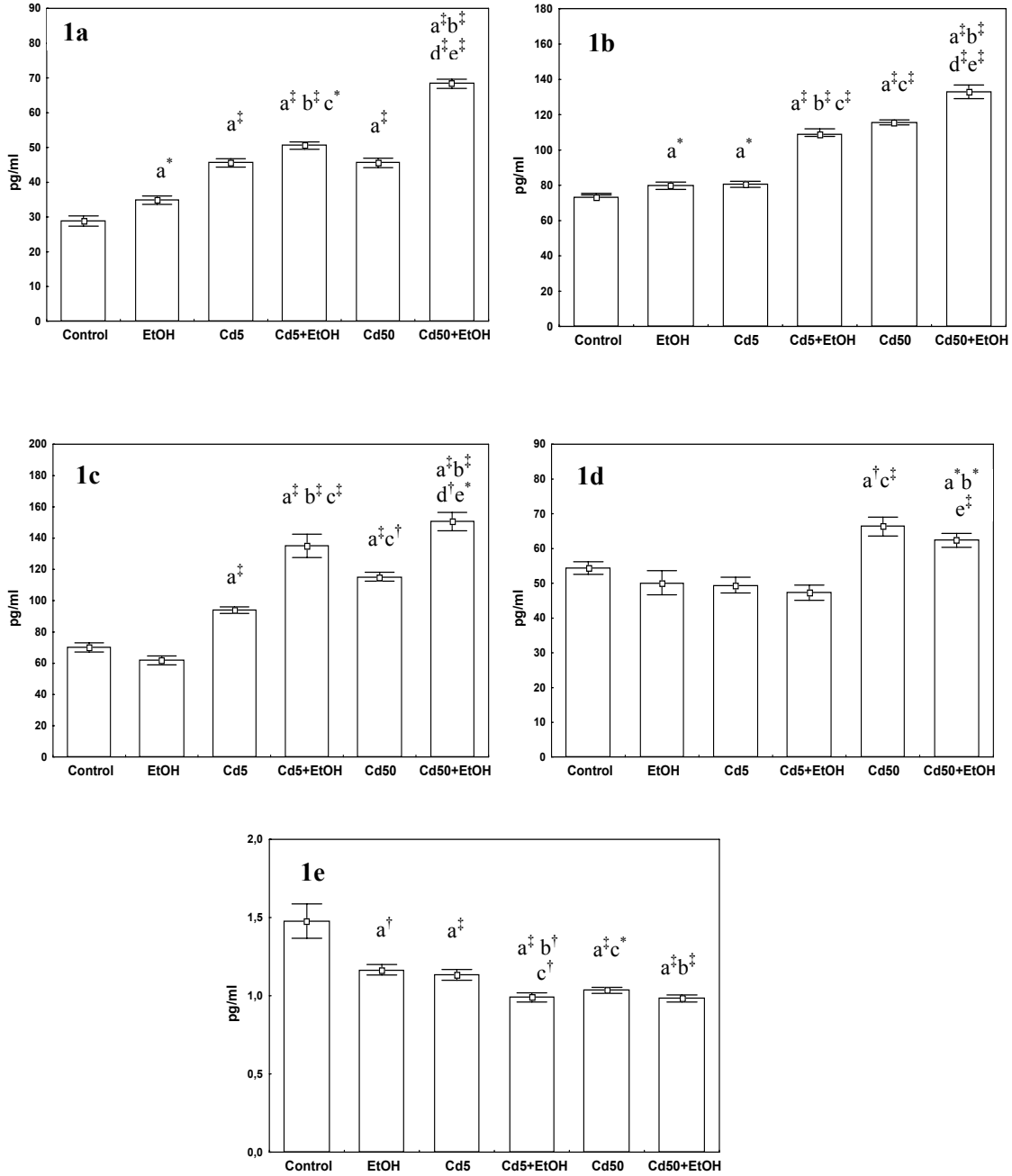

Fig. 1. Tumor necrosis factor-alfa (1a), interferon-gamma (1b), interleukin-1alfa (1c), interleukin-6 (1d) and interleukin-4 (1e) concentrations in the serum of rats exposed to cadmium (Cd) and/or ethanol (EtOH).
Data are mean ±S.E for 8 animals. Statistically significant differences (ANOVA, Duncan's multiple range test) are indicated by: [a] vs. control; [b] vs. EtOH group; [c] vs. Cd5 group; [d] vs. Cd50 group; [e] vs. Cd5+EtOH group. * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$.

(IFNγ), interleukin-1 (IL-1) and interleukin-6 (IL-6), through various cells of the immune system (T and B lymphocytes, monocytes, macrophages). However, until now there has been a lack of data regarding cytokine damage during co-exposure to both xenobiotics.

The detailed mechanisms of the effects of Cd or EtOH on cytokines are still unclear. It is postulated that those xenobiotics can influence the concentrations of inflammatory cytokines through oxidative stress mechanisms [27-29]. We have recently reported that exposure to Cd and/or EtOH stimulates oxidative stress [30-33].

Thus, the present study was aimed to investigate, on rat models of chronic moderate and relatively high human exposure to Cd (5 and 50 mg Cd/l in drinking water, respectively) and abuse of ethyl alcohol, whether exposure to Cd and EtOH, both alone and combined, may lead to damage to inflammatory cytokines, as well as to estimate whether and to what extent the effects of co-exposure differ from those observed under the treatment with each substance alone. For this purpose, the concentrations of TNFα, INFγ, IL-1α and IL-6 (pro-inflammatory cytokines) and interlekin-4 (IL-4, anti-inflammatory cytokine) in the serum of rats were determined. In order to estimate the involvement of the Cd- or/and EtOH-induced oxidative stress in their influence on the serum cytokine profile, the concentration of protein carbonyl groups (PC), as a marker of oxidative protein damage, was also determined. According to our knowledge, similar studies had not been conducted before.

Experimental Procedures

Animals and Treatment

A total of 48 young male Wistar rats (4-weeks-old, weighing approximately 70 g) were used. They were housed under controlled conventional conditions (temperature 22 ± 2ºC, relative humidity of 50 ± 10%, 12 h light:12 h dark cycle) and had free access to drinking water (redistilled water free of contamination) and a standard rodent laboratory LSM diet throughout the experiment. The energetic value of the diet was 12.2 MJ/kg. Cd concentration (assessed in our laboratory) in the food was 0.098 μg/g.

Throughout the experiment the animals were maintained in accordance with the institutional guidelines and international Guide for Use of Animals in Biomedical Research. The experimental protocol was approved by the Local Ethics Committee for Animal Experiments in Białystok, Poland.

The rats were randomly divided into six groups of 8 animals each: control group, one group receiving EtOH (EtOH group), two groups treated with cadmium chloride ($CdCl_2$) at the concentration of Cd^{2+} ion of 5 mg/l (Cd5 group) or 50 mg/l (Cd50 group), and two groups receiving Cd and EtOH in combination (Cd5+EtOH and Cd50+EtOH groups). Cd was administered in drinking water as $CdCl_2$ x 2½ H_2O (POCh; Gliwice, Poland) for 16 weeks. When the rats were adult (8-weeks-old), EtOH was administered p.o. (intragastrically through a tube) in the total dose of 5 g/kg b.wt./24 h divided into two equal doses (2.5 g/kg b.wt. each; the first dose was administered at 8 a.m., the other 6 hours later) for five consecutive days a week (from Monday to Friday) for 12 weeks. EtOH was applied in the form of 40% solution prepared from rectified spirit (95% v/v; Polmos, Lublin, Poland). To administer the dose of 5 g/kg b.wt./24 h to each rat, the volume of EtOH solution was calculated individually for each animal of the EtOH, Cd5 + EtOH and Cd50 + EtOH groups, taking into consideration changes in body weight during the experimental period. Body weight of rats was monitored on the first day of each week and on this basis appropriate volume of the 40% EtOH solution was calculated for each animal. The control group was divided into 2 subgroups which, like the EtOH group, drank redistilled water. When the rats of the EtOH and Cd + EtOH groups received alcohol, one of the control subgroups was administered physiological saline per os.

At termination, after overnight fasting, whole blood was taken by cardiac puncture with and without anticoagulant (heparin) under anesthesia with Vetbutal (pentobarbital sodium : pentobarbital 5:1, 30 mg/kg b.wt., i.p.) from all rats of each group. A portion of the whole blood collected without anticoagulant centrifuged after coagulation and serum was separated. The serum not used immediately was frozen at -70ºC until further analysis.

The concentrations of TNFα, INFγ, IL-1α, IL-6, IL-4 and PC in the serum were determined. The whole blood was subjected to Cd analysis, but detailed results will be presented elsewhere.

To evaluate the daily intake of Cd in each of the experimental groups and express them as mg/kg b.wt., the 24-h consumption of drinking water and weekly body weight gain were monitored throughout the experiment. At both levels of exposure, the daily Cd doses were within the same ranges of values irrespective of whether administrated alone or in combination with EtOH. This is very important in the evaluation of interaction between two substances.

The mean (mean ± S.E) daily intakes of Cd during the 16-week experimental period in the rats treated with 5 and 50 mg Cd/l (alone or with EtOH) were 0.825 ± 0.064 mg/kg b.wt. and 7.518 ± 0.597 mg/kg b.wt. or 0.698 ± 0.077 mg/kg b.wt. and 6.442 ± 0.603 mg/kg b.wt., respectively.

Because, Cd absorption from the gastrointestinal tract in the rat is very low (only 0.2-2% [4, 34] and markedly lower than in humans [2], the animals need higher Cd doses to reach its blood concentration (indicator of exposure) similar to those noted in humans. Blood Cd concentration in rats treated with 5 mg Cd/l are within the range of values noted in persons inhabiting moderately polluted areas, especially those being cigarette smokers [4], whereas the concentration of Cd in the animals intoxicated with 50 mg Cd/l is comparable to those noted in subjects inhabiting heavily polluted areas or/and being occupationally exposed to this metal [35]. The blood Cd concentrations in the rats exposed in the present study to 5 and 50 mg Cd/l reached 0.694 ± 0.021 μg/l (mean ± S.E.) and 8.573 ± 0.364 μg/l (mean ± S.E.), respectively (data not published).

The applied daily dose of EtOH (5 g/kg b.wt.) is equivalent to its consumption that may take place among alcohol abusers. Taking into account 3 times faster EtOH oxidation in rats than in humans (0.1 g/kg b.wt./h) [36], the dose of 5 g EtOH/kg b.wt./24 h corresponds to the daily consumption of about 0.7 l of 40% vodka by an adult man (weighing about 80 kg).

Analytical Procedures

Cytokine Determination

The concentrations of IL-1α, IL-6, IL-4, TNFα and IFNγ in the serum were determined by enzyme immunoassay using ELISA kits from Bender MedSystems (Austria) according to the kit instruction. Optical density was measured spectrophotometrically using an automatic plate reader (ELx800, BIO-TEK INSTRUMENTS, INC, Winooski, USA) at 450 nm. The sensitivities of methods indicated in the instructions of enzyme immunoassay kits are 4.1 pg/ml for IL-1α, 12 pg/ml for IL-6, 0.2 pg/ml for IL-4, 11.2 pg/ml for TNFα and 9.9 pg/ml for INFγ. The intra-assay coefficient of variation (CV) for the measurements was <5%.

PC Determination

The concentration of PC in the serum was determined using the Protein Carbonyl Assay Kit by Cayman Chemical Company (Ann Arbor, MI, USA). The assay is based on the measurement of absorbance (at 360-385 nm) of the colour product of PC reaction with 2,4-dinitrophenylhydrazine (DNPH). The absorbance was read at 370 nm using ELISA universal microplate reader. The intra-assay coefficient of variation (CV) for the measurements was <5%. The concentration of PC in the serum was expressed as nmol/mg protein.

Protein Determination

In order to express the concentration of PC in calculation per total protein, its serum level was determined as described in the instruction for the Protein Carbonyl Assay Kit (Cayman Chemical Company). Bovine serum albumin, dissolved in guanidine, was used to prepare a standard curve.

Statistical Analysis

Since there were no differences in any of the studied parameters between the two control subgroups, the results have been presented together as one control group. Values are mean ± S.E. of eight rats in each group.

A one-way analysis of variance (ANOVA) was first performed to determine whether there were statistically significant ($p < 0.05$) differences among the experimental groups. Further, a multivariate analysis of variance with Duncan's range post hoc test was used for comparisons between individual groups and to determine which means differed statistically significantly ($p < 0.05$).

Spearman rank correlation analysis was performed to investigate the relationship among the parameters studied. Differences and correlations were considered statistically significant at $p < 0.05$.

The statistical calculations were made using a Statistica 6.0 package (StatSoft, Tulsa, OK, USA).

Results

Serum Concentrations of Cytokines

Exposure to Cd and EtOH, alone and in combination, caused an increase in TNFα concentration by 58% in the Cd5 group, by 58% in the Cd50 group, by 21% in the EtOH group and by 75% in the Cd5+EtOH group, as well as a twofold rise in the Cd50+EtOH group (Fig. 1a). The concentration of TNFα in the Cd5+EtOH group was elevated as compared to the Cd5 group and the EtOH group by 11% and 45%, respectively. The co-exposure to 50 mg Cd/l and EtOH (the Cd50 + EtOH group) led to an increase in TNFα concentration as compared to the Cd50 group (by 50%) and the EtOH group (by 96%). In the Cd50+EtOH group, the concentration of TNFα was significantly higher (by 35%) than in the Cd5+EtOH group (Fig. 1a).

In comparison to the control group, the serum concentrations of INFγ was found to increase by 10% in the Cd5 group and by 57% in the Cd50 group (Fig. 1b). After the administration of EtOH alone, the concentration of INFγ was 9% higher than in the control group. Simultaneous exposure to 5 mg Cd/l and EtOH (Cd5+EtOH group) increased INFγ concentration by 49% as compared to the control group, by 36% in comparison to the Cd5 group and by 38% compared to the EtOH group. The concentration of INFγ in the Cd50+EtOH group increased by 81%, 15%, 67% and 21% compared to the control, Cd50, EtOH and Cd5+EtOH groups, respectively (Fig. 1b).

In rats exposed to Cd, both alone and in combination with EtOH, an increase in the serum concentrations of IL-1α by 34% (Cd5 group), 64% (Cd50 group) and 93% (Cd5 + EtOH group), and a twofold rise in the Cd50 + EtOH group as compared to the control group were observed (Fig. 1c). The concentration of IL-1α in the Cd5 + EtOH group was elevated by 44% in comparison to the Cd5 group and was twice as high as in the EtOH group. In the Cd50+EtOH group, the concentration of IL-1α was higher by 31% and 11% as compared to the Cd50 group and the Cd5 + EtOH group, respectively, and twice as high as in the EtOH group. In the Cd50 group, the concentration of IL-1α was higher by 23% than in the Cd5 group. Administration of EtOH alone had no effect on the serum concentration of IL-α (Fig. 1c).

Exposure of rats to 5 mg Cd/l, alone or in combination with EtOH, and to EtOH alone had no effect on the serum concentration of IL-6 (Fig. 1d). In the Cd50 group, the concentration of IL-6 was elevated by 21% as compared to the control group and by 34% in comparison to the Cd5 group. In the Cd50 + EtOH group, the concentration of IL-6 was higher by 15% than in the control group, by 24% than in the

Table 1. Correlation coefficients between the studied parameters in the serum[a].

Parameters	IL-1α	IL-6	IL-4	TNFα	INFγ
PC	r = 0.554 ‡	r = 0.403 †	r = - 0.503‡	r = 0.604 ‡	r = 0.786 ‡
IL-1α		r = 0.226 §	r = - 0.730 ‡	r = 0.845 ‡	r = 0.796 ‡
IL-6			r = - 0.197 §	r = 0.256 §	r = 0.349 *
IL-4				r = - 0.688 ‡	r = - 0.697 ‡
TNFα					r = 0.786 ‡

PC – protein carbonyl groups; IL-1α - interleukin 1-alfa; IL-6 – interleukin-6; IL-4 – interleukin-4; TNFα - tumor necrosis factor - alpha; INFγ - interferon-gamma.

[a] Spearman rank correlation analysis was conduced to investigate the relationship between variables. Data are presented as correlation coefficients (r) and the level of statistically significance (p)

* $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$; § $p > 0.05$.

EtOH group and by 32% in comparison to the Cd5 + EtOH group (Fig. 1d).

Separate and simultaneous administration of Cd and EtOH caused a decrease in the serum concentration of IL-4 by 23% in the Cd5 group, by 30% in the Cd50 group, by 21% in the EtOH group, by 33% in the Cd5+EtOH group and by 33% in the Cd50 + EtOH group as compared to the control group (Fig. 1e). The concentration of IL-4 in the Cd5 + EtOH group was lower by 13% than in the Cd5 group and by 15% than in the EtOH group. Exposure to 50 mg Cd/l (Cd50 group) caused a 9% decrease in the concentration of IL-4 as compared to the Cd5 group. In the Cd50 + EtOH group the concentration of IL-4 was decreased by 16% in comparison to the EtOH group (Fig. 1e).

Serum Concentrations of PC

The administration of 5 amd 50 mg Cd/l alone caused an increase in the serum concentration of PC by 43% and 94%, respectively, compared to the control group. (Fig. 2). Intoxication with EtOH resulted in a 70% increase in the concentration of PC. The co-exposure to Cd and EtOH led to an increase in PC concentration by 57% in the Cd5 + EtOH group and caused its twofold rise in the Cd50 + EtOH group in comparison to the control group. In the group of rats exposed to a combined action of higher dose of cadmium and EtOH (Cd50 + EtOH), the increase in the serum concentration of PC was also significantly higher than in rats exposed to either Cd or EtOH by 19% and 35% in the Cd50 and EtOH group, respectively. In the Cd50 + EtOH group the concentration of PC was significantly higher (by 47%) than in the Cd5+EtOH group (Fig. 2).

Mutual Correlations between Serum Concentrations of the Measured Parameters

The serum concentration of PC positively correlated with the concentrations of IL-1α, IL-6, TNFα and INFγ, and negatively with the concentration of IL-4 (Table 1). The serum concentration of IL-4 also negatively correlated with the concentrations of IL-1α, TNFα and INFγ. Moreover, positive correlations were noted between IL-1α concentration and the concentrations of TNFα and INFγ, as well as between INFγ concentration and the concentrations of IL-6 and TNFα (Table 1).

Discussion

This paper is the first report to investigate the concentrations of pro-inflammatory cytokines (TNFα, INFγ, IL-1α, IL-6) and anti-inflammatory cytokine (IL-4) during simultaneous exposure to Cd and EtOH, and reveal that at co-exposure the damage to some cytokines were more advanced than the treatment with each substance alone, especially higher Cd dosage. Moreover, the results suggest that oxidative stress may be involved in the mechanism leading to changes in the serum concentration of cytokines after exposure to Cd and EtOH alone and in combination.

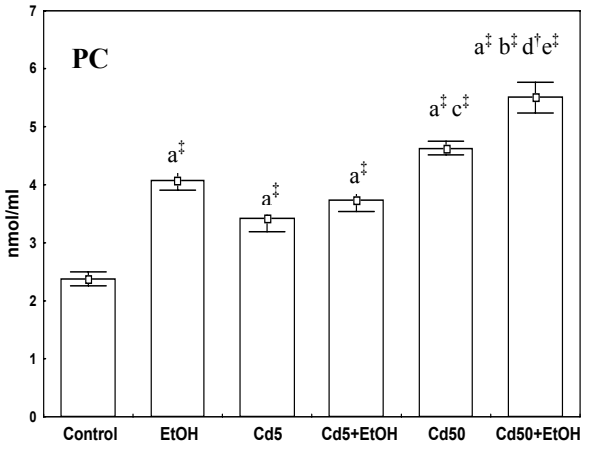

Fig. 2. Protein carbonyl group (PC) concentrations in the serum of rats exposed to cadmium (Cd) and/or ethanol (EtOH).
Data are mean ± S.E for 8 animals. Statistically significant differences (ANOVA, Duncan's multiple range test) are indicated by: [a] vs. control; [b] vs. EtOH group; [c] vs. Cd5 group; [d] vs. Cd50 group; [e] vs. Cd5+EtOH group. † $p < 0.01$; ‡ $p < 0.001$.

The changes observed in the present study in the concentrations of TNFα, INFγ, IL-1α, IL-6 and IL-4 in the serum rats exposed to Cd and EtOH may be due to the immunomodulatory actions of these xenobiotics on the immune cells.

There exists a strict relationship between TNFα, INFγ, IL-1α and IL-6 [37, 38]. IL-1α, which activates the innate and adaptive immune responses, stimulates the production of INFγ by T lymphocytes [39]. INFγ can induce IL-1α expression and enhance the cytotoxic action of TNFα [38]. TNFα is a major cytokine of the inflammatory and immune responses. TNFα induces the secretion of INFγ by lymphocytes and IL-1α by macrophages [37, 40]. IL-6 is a pleiotropic cytokine produced by monocytes, macrophages and by epithelial cells of the renal tubule [41]. One of the factors inducing its production is INFγ. Moreover, these cytokines can be produced by the same immune cells (e.g. macrophages, monocytes, T lymphocytes) and exert a simultaneous effect on the same cells. The high positive corelations observed in the present study between these cytokines seems to confirm the relationships between them.

IL-4 is mainly generated by Th2 lymphocytes and is involved in the humoral response [42]. The concentration of this cytokine in the organism is strictly connected with the production of INFγ being a factor inhibiting its secretion. The negative correlation noted between IL-4 concentration and the concentrations of INFγ and TNFα can suggest that the decrease in the serum concentration of IL-4 noted in rats exposed to Cd or/and EtOH might be a result of negative feedback.

Other authors have also reported that exposure to Cd stimulates the production of TNFα, INFγ, IL-1α and IL-6 [15, 23, 43]. Kataranowski et al. [15] have revealed that exposure of rats to Cd led to disorders in the cellular immune response characterized by qualitative and quantitative changes in granulocytes and elevated serum concentrations of TNFα, and IL-6. Yucesoy et al. [23] have found elevated concentrations of INFγ in the serum of workers occupationally exposed to Cd. Krocova et al. [43], assessing the immunomodulatory effect of Cd in the culture of the spleen cells, showed increased concentrations of IL-1α and INFγ in the medium.

The modulating effect of Cd on the expression of cytokines have been investigated in several *in vivo* and *in vitro* models; however, not all results are consistent and the mechanisms of the action are still unclear [44]. It has been postulated that Cd can influence the concentrations of cytokines by involvement in the induction of oxidative stress [27].

The available literature data show that EtOH-induced disorders in the humoral and cellular immune responses may result from the influence of EtOH on the production of cytokines [16, 26]. In our study, the increase in TNFα and INFγ concentrations due to EtOH alone was accompanied by simultaneous decrease in the concentration of IL-4. As in exposure to Cd, the decrease in IL-4 concentration may result from the elevated concentrations of TNFα and INFγ. TNFα is one of the best known cytokines. It is produced most frequently by monocytes-macrophages after their stimulation, especially in response to bacterial endotoxins (lipopolysaccharide, LPS). Węglarz et al. [20, 21] have revealed that LPS, released from the bacterial surface in the intestinal strain, plays an important role in the secretion of TNFα by human mononuclear cells. EtOH damaging the intestinal barrier accelerates the absorption of endotoxins (including LPS) from the intestinal lumen into circulation. Endotoxaemia induced by this xenobiotic activates Kupffer cells (stationary hepatic macrophages) to enhanced production of TNFα, IL-1, IL-6 and IL-8 [17, 28, 29, 45] as well as free radicals [28]. The change in the serum concentration of TNFα in the EtOH group is, in general, consistent with findings of other authors [46, 47]. Lin et al. [46] and Worall et al. [47] have noted an increase in the serum concentration of TNFα due to EtOH administration for 6 weeks. Moreover, unlike us, Worall et al. [47] have also observed an increase in the concentration of IL-6.

The mechanism by which EtOH exerts its modulatory effects on immune cells is not well understood. It has been suggested that alterations in membrane fluidity [48], protein phosphorylation, or protein-protein interactions ultimately result in changes of signalling transduction within the cell [49, 50]. Literature data give evidence that EtOH-induced oxidative stress may be involved in the stimulation of cytokine production [28, 51]. Many reports are consistent with the hypothesis that EtOH-induced reactive oxygen species (ROS) not only act as toxic substances, but also stimulate signal transduction by activating redox-sensitive nuclear transcription factor (NF-κB), which in turn leads to TNFα production [52, 53].

EtOH administered to the rats exposed to Cd from infancy enhanced the Cd-induced changes in the concentrations of TNFα, INFγ and IL-1α. The modyfing effect of EtOH was especially pronounced at the higher level of Cd exposure. The mechanisms which intensify the changes in cytokine concentration under combined exposure to Cd and EtOH are unknown. Simultaneous exposure to these xenobiotics potentiates changes in the serum profile of cytokines, mainly TNFα, and this effect may result from oxidative stress induced by their co-administration. We have recently reported that Cd or/and EtOH stimulate oxidative stress [30-33].
The statistically significant correlations noted between the serum concentration of PC, reflecting oxidative protein damage, and the concentrations of TNFα, INFγ, IL-1α, IL-6 and IL-4, may confirm the involvement of oxidative stress in the mechanism of influence of Cd and/or EtOH on the serum profile of these cytokines.

Conclusions

Based on the results of the present study it can be concluded that co-exposure to Cd or EtOH may cause more advanced changes in the serum profile of inflammatory cytokines than treatment with each substance alone. Moreover, the results seem to suggest that oxidative stress may be involved in the mechanism of the Cd- and/or EtOH-induced changes in the concentration of cytokines.

The findings of the present study allow us to hypothesize that subjects exposed to Cd and EtOH, and especially those co-exposed to both substances, may show lower resistance for factors inducing inflammatory processes, including bacteria and viruses.

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