Influences of DTC and zinc supplementation on the cellular response restoration in restrained mice

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The studies were conducted on Balb/c mice exposed to restraint stress twice for 12 h at 24 h intervals. Prior to restraint stress the mice were treated with sodium diethyldithiocarbamate (DTC) i.p. at a dose of 20 mg/kg five times at 48 h intervals. DTC was used per se or with zinc ions interaction, by adding zinc sulfate to drinking water at a dose of 72 µg/mouse daily. The results obtained in the study show that restraint stress causes involution of lymphatic organs, decreased the percentage of immature (CD4+CD8–) and, mature (CD4+) thymocytes and CD4+, CD8+ and CD19+ splenocytes and proliferative response of thymocytes stimulated in vitro with concanavalin A (Con A) and phytohemagglutinin (PHA). The restraint stress decreased also interleukin-1 (IL-1) production by murine intraperitoneal macrophages stimulated in vitro with lipopolysaccharide (LPS) from E. coli. Pretreatment with DTC counteracted restraint stress-induced immunosuppression, which is expressed as partial normalisation of the total number of thymocytes, splenocytes and IL-1 production, accelerated regeneration of thymus and spleen, shorter suppressive action of restraint stress on the percentage of CD4+CD8– thymocytes and in total normalisation of the CD4+ thymocytes and splenocytes. DTC administered prior to restraint stress augmented the proliferative response of thymocytes to two mitogens. The immunocorrecting action of DTC is enhanced by zinc supplementation, expressed in the increased percentage of CD4+ thymocytes and splenocytes, CD19+ splenocytes, proliferative activity of thymocytes stimulated with PHA and IL-1 production. The obtained results show that DTC administration can be supplemented with zinc in order to restore the immune system impaired by stress.

**Key words:** DTC, zinc ions, restraint stress, cellular immune response, mice

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**Introduction**

Sodium diethyldithiocarbamate (DTC) is a synthetic immunomodulator belonging to class I thymomimetic drugs, accelerating maturation and differentiation of prothymocytes and modulating the functions of mature T lymphocytes [21]. The effect of DTC is associated with the stimulation of hepatocytes to synthetize and release the serum thymic hormone-like factor, directly and indirectly mediated by the central nervous system [21,24]. It is known that this serum factor can be transferred *in vivo* and *in vitro* and stimulates differentiation of thymocytes [25]. The studies of Renoux and Renoux [22] show that the DTC-induced serum factor was demonstrated in young mice as well as in nude mice to stimulate precursor cells to differentiate into T cells, then trigger the different steps of T cell maturation. Presumably, the modulating action of DTC is connected not only with the induction of the markers of T lymphocyte differentiation, but also with the effect of this drug on T lymphocyte differentiation, but also with the effect of this drug on T lymphocyte and macrophage functions by stimulating the production of interleukin-2 (IL-2), interferon-γ (IFN-γ) and interleukin-1(II-1) [3].

Zinc is a crucial nutritional component required for normal development and maintenance of immune functions. It has been found that zinc acts as an inhibitor of apoptotic cell death and plays a more complex role in physiological intrathymic cell selection [19,28]. The thymus is an organ responsible for providing the immunocompetent peripheral cells with zinc ions and this depends on the concentration of zinc ions in serum. Zinc ions in epithelial cells form complexes with thymulin and thymosine-α, which together with IL-1, interleukin-6 (IL-6) and interleukin-7 (IL-7) are responsible for intra- and extrathymic differentiation and maturation of T lymphocytes [8,27]. The cardinal sign of zinc deficiency is thymic involution, which subsequently attenuates the activity of immunocompetent cells, notably T lymphocytes, macrophages and natural killer cells [9,11].

The immunosuppressive effect of acute stress is connected with a markedly increased catecholamines levels in blood and augmented glucocorticoid production resulting from
stimulation of the hypothalamic-pituitary-adrenal axis [6,10]. The increased level of glucocorticoids induces the apoptosis of immature double-positive thymocytes and suppress the endocrine activity of thymic epithelial cells, consequently reducing differentiation and maturation of thymocytes [5,7].

The purpose of the present study was to determine the ability of DTC in combination with zinc supplementation to restore the cellular immune response impaired by the exposure of mice to restraint stress. It has been found that acute stress results in the involution of thymus, which subsequently attenuates cellular and humoral response although the latter to a lesser degree.

**Material and Methods**

**Animals**

The studies were conducted on male and female Balb/c mice, each weighing 15-17 g (5-6 weeks of age). The experimental animals were obtained from a breeding laboratory at the Medical University, Wroclaw, Poland. Principles of laboratory animal care (NIH publication No 86-23, revised 1985), as well as the specific national laws on the protection of animals were followed.

The mice were exposed to restraint stress twice at 24 h intervals. For this purpose they were kept in restraint cages (specially prepared for the purpose of the study) for 12 hours (from 9 p.m. to 9 a.m.). At the same time, the control mice were allowed to remain in their home cages, but they had no access to food and water during the stress period of their counterparts [30]. Each experimental group consisted of eight mice.

**Drugs and treatment**

Sodium diethyldithiocarbamate (DTC in subst. purified and recrystallized; Poch, Poland) at a dose of 20 mg/kg were dissolved in phosphate buffered saline (PBS) and injected to mice intraperitoneally, five times at 48 h intervals, prior to stress exposure. The volume of DTC was 0.2 ml/mouse. Oral zinc supplementation was performed by the administration of zinc sulfate (ZnSO₄, 7H₂O; Ciech, Poland) dissolved in tap water. Zinc ions (as sulphate salt) at a dose of 72 µg/day per mouse were administered orally for 10 days prior to restrained stress exposure. Mice in the control group were treated with PBS (0.2 ml/mouse).

**Measurements**

The determinations included: (i) the total number of thymocytes and splenocytes; (ii) the weight ratio of thymus and spleen calculated according to the following formula: weight of organ (mg)/body weight of mouse (mg) × 100; (iii) proliferative response of thymocytes stimulated *in vitro* with concanavalin A (Con A; Serva, Germany) or phytohemagglutinin (PHA; Serva, Germany) according to the method described by Bradley [1]; (iv) CD subsets (CD4⁺, CD8⁺ and CD4⁺CD8⁺) in thymus and CD4⁺, CD8⁺ and CD19⁺ in spleen were determined by direct immunofluorescence assay using monoclonal antibodies (mAb) coupled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE); (v) the production of IL-1 in the culture supernatants of peritoneal macrophages stimulated with lipopolysaccharide from *Escherichia coli* (LPS) were determined by means of an ELISA kit for determination of murine IL-1β (R&D Systems, USA).

The total number of thymocytes, splenocytes, weight ratios of the thymus and spleen, proliferative response of thymocytes non-stimulated or stimulated with Con A or PHA and CD subsets of thymocytes and splenocytes were determined four times: immediately after the stress exposure was ended and on days 2, 5 and 10 following the exposure to restraint stress. The production of IL-1 was determined once, immediately after the stress exposure.

**Mitogen responsiveness**

The mice anesthetized with halothane were sacrificed and thereafter the thymuses were removed in a sterile manner. The thymocyte suspension (2 × 10⁶/ml) was prepared in the RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Serva, Germany), and L-glutamine (Serva, Germany) at a concentration of 30 mg/500 ml, and gentamycin (Sigma, USA) at a concentration of 50 mg/500 ml of the medium. The viability of each thymocyte suspension was determined by trypan blue dye exclusion. It was found at the level 90-95%.

Mitogenic response was assessed in 96-well plastic microtitre plates (Costar, USA) containing 5 × 10⁴ thymocytes in 0.2 ml of RPMI 1640 medium supplemented with 10% FCS, gentamycin and L-glutamine in the presence of an optimal concentration of Con A (5 µg/ml) or PHA (5 µg/ml). The thymocyte cultures were incubated at 37°C for 48 h in a humidified atmosphere of 5% CO₂ and 95% air. After 24 h of incubation, tritiated thymidine (6-³H-thymidine, Institute for Research, Production and Application of Radioisotopes, Czech) 40 Hbg/ml (1 µCi/200 µl) was added to the culture. The culture was harvested 24 h later onto paper filters using a cell harvester (Skatron, Norway) and the incorporated thymidine was counted using a liquid scintillation counter (Packard Instruments, USA). The data from quadruplicate cultures were expressed as mean counts per minute plus or minus the standard error of the mean (cpm ± SE).

**Assay of thymocyte and splenocyte subsets**

Mice were anaesthetized with halothane after the restrained stress exposure. The thymuses and spleens were removed and placed in disposable Petri dishes containing sterile, ice-cold PBS. The suspended cells were released from the lymphatic organs by passage through a nylon mesh.
and then centrifuged on a layer of Ficoll 400 (Pharmacia, Sweden)/Uropolinum 75% (diazotize sodium and meglumine diazotize, Polpharma, Poland) in 1:3 ratio, density 1.071. After centrifugation at 4°C cells were collected from the interphase and washed twice with PBS supplemented with 1% bovine serum albumine (BSA; Sigma, USA) at 4°C. After the second wash cells were suspended in PBS with 1% BSA at 1 × 10^6 cells/ml. The viability of each cell suspension was determined by trypan blue exclusion. It was found that at level 90-98%. Cells were resuspended in 100 µl PBS buffer with 1% BSA and stained with FITC-labelled antibody to mouse CD4 clone: YTS 177.9 (lot: 14218-02S; BioSource, USA) and PE-labelled antibody to mouse CD8 clone: KT15 (lot: 13927-03S, BioSource, USA) or PE-labelled antibody to mouse CD19 clone: 6D5 (lot: 16249-02S; BioSource, USA) in a dilution recommended by the producers. Cells were incubated at 4°C for 30 min., and washed three times with ice-cold PBS buffer and resuspended in 50 µl PBS buffer and microscope preparations were made. Using an Axiosplan fluorescence microscope (Opton, Austria) CD4+, CD8+, CD4CD8+ thymocyte levels and CD4+, CD8+ and CD19+ splenocyte levels were determined, each time scoring 300 cells.

**Production of interleukin-1 (IL-1)**

Mice were anaesthetized with halothane. Peritoneal exudate macrophages were harvested in sterile, ice-cold phosphate buffered saline solution (PBS) with antibiotics (penicillin G 10 U/ml and streptomycin 1 µg/ml, Sigma, USA). Cells were washed and suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Flow Lab, USA), 10 mM HEPES (Sigma, USA), 2 mM L-glutamine (Sigma, USA) and antibiotics (penicillin G 10 U/ml and streptomycin 1 µg/ml, Sigma, USA), adjusted to a concentration of 1.5 × 10^6 cells/ml, dispensed in 100 ml volumes in 96-well flat bottom plate (Costar, USA). The medium with nonadherent cells was replaced after 3 h incubation at 37°C in normal atmosphere with 5% CO₂. Incubation was continued and the medium was replaced after 18 h by the medium without FCS, but containing LPS from *E. coli* (O55:B5; Sigma, USA) at a concentration of 2.5 µg/ml. Each culture was tested in triplicate. After 24 h of incubation, supernatants were removed and stored at −70°C. A commercial ELISA kit (R&D Systems, USA) was used to determine mouse IL-1β in macrophage culture supernatants, according to the manufacturers instructions.

**Statistical analysis**

The data obtained in the study were analysed statistically using a Student t-test. The differences were considered significant at 5% (*p < 0.05*).

**Results**

The effects of DTC with zinc ions interaction on the total number of thymocytes and splenocytes and weight of the thymus and spleen in restrained mice

As reported in Table 1, the total number of thymocytes and splenocytes and the weight ratio of thymus and spleen of restrained mice markedly decreased as early as 24 h following the exposure to stress. The suppressive effect of acute stress sustained for 10 days of the observation. DTC administered to mice prior to stress exposure partially

| Table 1. Total number of thymocytes, splenocytes and weight ratio of thymus and spleen in restrained mice treated with DTC and zinc supplementation prior to stress (mean±SD) |
| Index | Day | Control | Stress | DTC+stress | Zn⁺+stress | DTC+Zn⁺+stress |
|-------|-----|---------|--------|------------|------------|----------------|
| Total number of thymocytes (n × 10⁶ cells) | 1 | 23.7±2.4 | 11.1±1.0* | 10.3±2.7* | 7.3±1.3* | 10.6±1.3* |
| | 2 | 23.2±2.7 | 6.5±1.1* | 8.5±1.2* | 8.5±1.4* | 12.2±1.1* |
| | 5 | 22.8±2.8 | 8.5±1.7* | 12.6±1.6* | 11.1±4.5* | 14.5±1.9* |
| | 10 | 21.1±2.2 | 15.0±3.0* | 18.5±2.9* | 14.2±3.3* | 21.2±1.8* |
| Weight ratio of thymus | 1 | 4.24±0.04 | 0.137±0.03* | 0.257±0.04* | 0.172±0.04* | 0.257±0.08* |
| | 2 | 0.407±0.03 | 0.135±0.02* | 0.258±0.02* | 0.189±0.04* | 0.260±0.04* |
| | 5 | 0.402±0.06 | 0.152±0.02* | 0.212±0.03* | 0.135±0.02* | 0.228±0.03* |
| | 10 | 0.355±0.08 | 0.213±0.03* | 0.383±0.06* | 0.216±0.06* | 0.374±0.05* |
| Total number of splenocytes (n × 10⁶ cells) | 1 | 30.7±2.4 | 11.1±1.6* | 13.2±1.7* | 11.3±1.8* | 11.2±1.9* |
| | 2 | 28.8±2.7 | 9.1±2.4* | 14.7±1.9* | 10.6±2.0* | 13.7±2.5* |
| | 5 | 30.5±3.0 | 15.2±4.1* | 21.5±2.7* | 14.0±3.2* | 22.2±3.3* |
| | 10 | 30.5±4.6 | 18.6±5.8* | 25.1±2.9* | 16.4±2.7* | 21.5±2.4* |
| Weight ratio of spleen | 1 | 0.683±0.08 | 0.471±0.05* | 0.557±0.07* | 0.483±0.16* | 0.496±0.05* |
| | 2 | 0.721±0.12 | 0.472±0.11* | 0.677±0.08* | 0.427±0.04* | 0.656±0.09* |
| | 5 | 0.706±0.09 | 0.537±0.09* | 0.681±0.12 | 0.427±0.15* | 0.736±0.18* |
| | 10 | 0.719±0.09 | 0.605±0.09* | 0.747±0.07* | 0.631±0.08* | 0.774±0.09* |

* *p<0.05 as compared to the control group.

* *p<0.05 as compared to the stress group.
counteracted the suppressive effect of stress on the total number of thymic and spleen cells. Pretreatment with DTC restore the weight ratio of the thymus and spleen to the control values after day 5 following the exposure to stress. Simultaneous administration of DTC and zinc ions did not change protective effect of DTC on the two lymphatic organs.

The effects of DTC with zinc ions interaction on mitogen-induced proliferation o thymocytes in restrained mice

As shown in Fig. 1 and 2, restraint stress markedly inhibited the proliferation of thymocytes stimulated in vitro with Con A and PHA as early as 24 h following the exposure. The decreased proliferative response of thymocytes to stimulation in vitro with PHA was maintained for 2 days, and on day 5 returned to the control value. On day 5 following stress exposure the proliferative response of thymocytes to Con A was higher than of the control, but on day 10 its value decreased again. Pretreatment with DTC totally abrogates the suppressive effect of restraint stress on the proliferative response of thymocytes to Con A (days 1 and 10) and also potentiates the response of the examined cells to this mitogen (days 2 and 5) paradoxically stimulated by stress on day 5 (Fig. 1). DTC did not change the inhibitory effect of restraint stress on the proliferative response of thymocytes to PHA, especially during the first day after exposure. However, administration of DTC prior to restraint stress augments the proliferative response to PHA between days 2 to 10 following the exposure to stress (Fig. 2). Oral zinc administration for 10 days prior to restraint stress did not change the effect of stress on the proliferative response of thymocytes stimulated in vitro with Con A, but totally prevents the suppressive effect of stress on the proliferative response of thymic cells to PHA. The combination of zinc ions and DTC not only counteracted the suppressive action of stress on the proliferative response to PHA, but also enhanced the stimulating action of DTC on the proliferative response to this mitogen between days 2 and 5 after the stress was finished (Fig. 2).

The effects of DTC and zinc ions supplementation on thymocyte and splenocyte subpopulations in restrained mice

As reported in Table 2 restraint stress decreased the percentage of immature CD4+CD8+ thymic cells (double-positive cells). The suppressive effect of stress sustained for 10 days of the observation. The lowest percentage of CD4+CD8+ thymocytes were observed between days 1 and 5 following exposure to stress. In contrast, only 1 day after exposure to stress a temporary decrease in the percentage of mature CD4+ thymocytes (single-positive cells) was observed, but no effect on CD8+ was found. At the same time, some changes in the percentage of the splenocyte subpopulations were found. Exposure to restraint stress decreased the percentage of CD4+CD8+ splenocytes (helper-inducer T cells), CD8+ splenocytes (suppressive and cytotoxic T cells) and CD19+ (B cells). The suppressive action of restraint stress on the percentage of CD4+ and CD19+ splenocyte subpopulations was maintained for 10 days. In addition, on days 2 and 5 following exposure to stress, a temporary decrease in the percentage of CD8+ splenocytes was observed. DTC administration prior to restraint stress totally counteracted the suppressive effect of stress on the single-positive thymocytes with CD4+ receptors and markedly reduced the inhibitory effect of stress on the percentage of immature, double-positive CD4+CD8+ thymic cells and CD4+ and CD8+ splenocytes. During a 10 day observation period DTC did not change the suppressive
action of restraint stress on the percentage of CD19+ splenocytes (B cells).

Administration of zinc ions prior to exposure to stress reduces the suppression and length of the stressor’s action on the percentage of the double-positive thymocytes, single-positive CD4+ thymic cells, CD4+ and CD8+ splenocytes. However, zinc ions did not change the suppressive effect of restraint stress on the percentage of CD19+ splenocytes.

The combination of zinc ions with DTC totally counteracted the suppressive action of restraint stress on the percentage of CD4+ and CD8+ splenocytes and accelerated regeneration of the thymus, which was expressed in faster recovery of the percentage of immature thymocytes to the control values. In addition, administration of DTC with zinc ions prior to exposure to stress not only counteracted the suppressive effect of stress on the percentage of CD4+ thymocytes and splenocytes, but also augmented the percentage of these cells for 10 days after the stress was completed. The combination of DTC with zinc ions administered to mice prior to stress exposure partially prevented the suppressive effect of stress on the percentage of CD19+ splenocytes during 5 days following the exposure.

The effects of DTC with zinc ions interaction on IL-1 production by intraperitoneal macrophages in restrained mice

Exposure to restraint stress decreases IL-1 production by intraperitoneal macrophages stimulated in vitro with LPS (2.5 µg/ml). Administration of DTC and zinc per se prior to restraint stress partially prevents the suppressive effect of stress on IL-1 production. In contrast, simultaneous administration of DTC with zinc before exposure to restraint stress totally counteracts the suppressive action of stress on IL-1 production by intraperitoneal macrophages in mice (Fig. 3).

### Discussion

The present study indicates that the administration of DTC (drug affecting the differentiation and maturation of T lymphocytes) prior to the exposure of mice to restraint stress partially or totally counteracts stress-induced immunosuppression. The protective or immunomodulating action of DTC is reflected in the accelerated process of thymus gland and spleen size reversion, restoration of the total number of cells of these two lymphatic organs, the percentage of CD4+

### Table 2. Percentage of thymocyte and splenocyte subpopulations in restrained mice treated with DTC and zinc supplementation prior to stress (mean±SD)

| Index     | Day | Control     | Stress | DTC+stress | Zn+stress | DTC+Zn+stress |
|-----------|-----|-------------|--------|------------|-----------|---------------|
| CD4+CD8+  | 1   | 74.1±4.0    | 47.1±7.1* | 51.7±4.7*  | 51.2±8.8*  | 63.5±5.0**    |
|           | 2   | 75.6±2.8    | 47.8±6.4* | 56.4±6.9*  | 48.4±3.9*  | 64.5±5.3**    |
|           | 5   | 76.3±4.5    | 50.5±2.5* | 61.8±6.2** | 58.3±4.8** | 60.6±2.9**    |
|           | 10  | 73.7±2.6    | 64.3±4.0* | 73.5±5.1*  | 70.1±3.9*  | 73.0±2.9*     |
| CD4+      | 1   | 15.1±1.7    | 11.3±2.3* | 15.8±2.0*  | 13.6±2.1  | 19.3±3.3**    |
|           | 2   | 13.2±2.3    | 13.5±2.8  | 14.5±2.3   | 13.0±2.5  | 18.8±2.6**    |
|           | 5   | 14.8±2.0    | 14.5±3.1  | 16.3±3.4*  | 14.1±3.6  | 19.2±2.1**    |
|           | 10  | 13.5±3.6    | 12.5±2.9  | 13.0±1.6   | 15.0±2.0  | 19.0±3.4**    |
| CD8+      | 1   | 4.8±1.7     | 3.7±1.4   | 4.2±1.8    | 4.7±1.4   | 4.3±1.9       |
|           | 2   | 4.7±1.1     | 4.3±1.5   | 3.7±2.1    | 4.5±2.5   | 4.7±2.2       |
|           | 5   | 4.4±1.4     | 3.9±1.4   | 5.8±1.1    | 4.8±1.2   | 5.8±1.5       |
|           | 10  | 4.6±1.0     | 4.5±1.2   | 5.1±2.1    | 5.0±1.5   | 3.2±1.8       |
| CD4+      | 1   | 19.5±2.3    | 11.2±2.0* | 20.2±4.4*  | 14.1±1.8* | 25.2±2.8**    |
| splenocytes| 2   | 18.9±3.5    | 12.0±1.9* | 18.1±3.5*  | 13.0±2.4* | 23.7±3.4**    |
|           | 5   | 21.9±1.9    | 12.3±1.6* | 18.7±2.1*  | 16.4±1.2* | 22.9±2.4**    |
|           | 10  | 20.8±3.3    | 13.2±1.6* | 21.6±4.6*  | 21.5±3.2* | 28.2±4.0**    |
| CD8+      | 1   | 12.2±2.0    | 10.3±2.0  | 10.7±1.9   | 10.4±1.8  | 10.3±2.0      |
| splenocytes| 2   | 12.6±1.6    | 4.5±1.2*  | 7.7±5.8**  | 8.4±2.1*  | 8.9±1.7**     |
|           | 5   | 12.7±1.9    | 7.8±2.6*  | 9.2±2.0*   | 9.0±2.2*  | 9.2±1.8*      |
|           | 10  | 10.8±2.0    | 9.2±1.9   | 9.8±1.7    | 9.8±1.8   | 9.2±2.1       |
| CD19+     | 1   | 50.1±4.3    | 31.9±6.2* | 37.1±6.6*  | 30.7±3.9* | 43.2±5.4**    |
| splenocytes| 2   | 48.1±1.9    | 24.6±4.0* | 28.3±5.8*  | 22.1±3.2* | 37.9±6.7**    |
|           | 5   | 48.7±4.5    | 20.7±2.8* | 24.7±2.4*  | 21.1±2.8* | 34.6±3.9**    |
|           | 10  | 49.8±3.1    | 43.1±3.1* | 43.7±3.7*  | 42.7±3.7* | 48.8±2.5*     |

*p<0.05 as compared to the control group.

*#p<0.05 as compared to the stress group.

#p<0.05 as compared to DTC+stress group.
thymocytes and splenocytes, and recovered proliferative activity of thymic cells stimulated in vitro with Con A and PHA.

It seems quite likely that immunocorrecting action of DTC is due not only to the induction of markers differentiating T lymphocytes, but also to the effect of the drug on T lymphocyte and macrophage functions by stimulating the synthesis and release of cytokines, such as IL-1, IL-2 or IFN-γ [3]. The results of the present study show that DTC administered prior to acute stress only partially counteracts the suppressive action of restraint stress on IL-1 production by peritoneal macrophages in mice. Earlier studies by the same author indicate that administration of DTC to restrained mice partially or totally restores humoral response of SRBC-immunized mice, depending on time of administration in relation to time of stress exposure [13]. It has been found that administration of DTC immediately after exposure of the mice to restraint stress totally restores their humoral response to the thymus-dependent antigen. Moreover, DTC was found to counteract the suppressive effect of cold stress and hypothermia on B lymphocytes producing haemolytic antibodies (PFC) and haemagglutinin levels in SRBC-immunized rabbits [17], which may suggest that DTC enhances the differentiation of helper-inducer T lymphocytes.

The results obtained in previous experiment conducted on mice show that administration of DTC at a dose of 20 mg/kg five times at 48 h intervals increases the percentage of mature CD4⁺ thymocytes with corresponding decreases in the percentage of immature CD4⁻CD8⁻ thymic cells (double positive cells) and also augments the percentage of CD4⁺ splenocytes, but does not affect the percentage of CD8⁺ thymocytes and splenocytes [15]. Other authors have also reported that DTC is able to restore functioning of the immune system impaired by prolonged administration of immunosuppressive drugs [23], and it is also capable of restoring the reactivity of some immunological responses impaired by ageing [2]. It has been found that DTC is able to partial restore the humoral response to SRBC in cyclophosphamide-suppressed mice [18] and also partially or totally counteracts the suppressive action of single, high hydrocortisone dose (125 mg/kg) on the percentage of T lymphocyte subpopulations, and proliferative activity of thymic cells stimulated in vitro with Con A and PHA [16].

The results of the present study show that the immunorestorative action of DTC is enhanced by zinc supplementation. It seems quite likely that zinc ions supplementation can modulate intra-thymic process of thymocyte differentiation and maturation. The experiments in vitro have shown that zinc ions inhibit apoptosis of murine thymocytes induced by dexamethasone added to cell culture [4]. At present it is assumed that the effect of zinc ions on glucocorticoid-induced apoptosis is connected with the inhibiting effect of this element on endonuclease activity, which prevents disruption of DNA into characteristic double-stranded fragments [5,29]. The results obtained in earlier study by the same authors indicate that pre-incubation of thymocytes with Zn²⁺ at concentration of 1-50 µg/ml/culture efficiently counteracts the cytotoxic effect of hydrocortisone on thymic cells. Besides zinc ions (1 µg/ml/culture) added simultaneously to the culture resulted in augmented preventive action of DTC against thymolytic action of hydrocortisone and increased the ranges of DTC concentrations, efficiently counteracting the cytotoxic action of hydrocortisone [14]. It has been also found that oral administration of zinc stimulates the epithelial thymic cells for producing zinc-thymomodulin complex which in combination with IL-1, IL-6 and IL-7 is responsible for intra- and extra-thymic differentiation and maturation of T lymphocytes [8,27]. In addition it has been found that administration of zinc or zinc-thymomodulin complex to mice augments the proliferative response of thymocytes and splenocytes stimulated in vitro with Con A, PHA, IL-1 and IL-2 [12,26]. The studies of Renoux et al. [20] indicate that administration of zinc-diethylthiocarbamate (Zn-DTC), depending on a dose, is able to increase the proliferative activity of murine splenocytes stimulated in vitro with Con A, PHA and PWN.

In conclusion, it can be stated that restraint stress causes involution of lymphatic organs (thymus and spleen) which is accompanied by decreased proliferative activity of thymocytes to Con A and PHA, the percentage of CD4⁺CD8⁻ and CD4⁺ thymocytes and CD4⁺, CD8⁻ and CD19⁺ splenocytes and inhibited IL-1 production by peritoneal macrophages. DTC administered prior to restraint stress partially or totally counteracts the suppressive effect of acute stress. The immunorestorative action of DTC is
potentiated by zinc supplementation. The results of the study indicate that thymomimetic drug such as DTC injection can be supplemented with oral zinc administration in order to restore the immune system impaired by environmental stressors.

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