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Original article

Developmental exposure to low doses of dichlorodiphenyltrichloroethane disrupts functional and morphologic maturation of the spleen in prepubertal rats

Bella B. Gagulaeva, Nataliya V. Yaglova, Sergey S. Obernikhin, Ekaterina P. Timokhina

Avtsyn Research Institute of Human Morphology of Petrovsky National Research Center of Surgery, Moscow, Russia

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Abstract: Dichlorodiphenyltrichloroethane (DDT) is a versatile persistent pollutant with endocrine disrupting properties and an alleged immune modulator.
Objective — to evaluate the parameters of functional and morphological maturation of the spleen in prepubertal rats exposed to low doses of DDT.
Material and Methods — From the moment of mating, during pregnancy and lactation, female rats received a solution of o,p-DDT (20 μg/L) instead of tap water. The offspring of intact rat dams were considered the control group. Male DDT-exposed and control rats were sacrificed on postnatal day 7. The spleens were surgically removed under sterile conditions. Anatomical and histological examination of the spleen, *ex tempore* proliferation of splenocytes, and assessment of splenic T cells and their ability to respond to the mitogen concanavalin A were assessed.
Results — Rats exposed to DDT exhibited a significantly reduced proliferative response of spleen lymphocytes to the mitogen concanavalin A. However, morphological evaluation revealed no differences in spleen anatomy between control and exposed rats. Histological examination exposed accelerated development of lymphoid tissue in the spleen of rats exposed to DDT. The ex-tempore proliferation test yielded a higher rate of mitotic division of splenocytes in exposed rats. In contrast to controls, they had a lower percentage of T cells in their spleen.
Conclusion — Developmental exposure to low doses of the endocrine disruptor DDT impairs functional and morphological maturation of the spleen in prepubertal rats. DDT accelerates the formation of lymphoid compartments and weakens the functional maturation of the spleen as the organ with an immune function.

Keywords: DDT, spleen, proliferative response, morphology.

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Correspondence to Nataliya V. Yaglova. E-mail: yaglova@mail.ru.

Introduction

According to recently published data, anthropogenic burden is associated with an increase in the incidence of immunological disorders, such as abnormal immune responses or autoimmune diseases. Exposure to various chemicals that disrupt the endocrine system is considered the most likely etiological and pathogenetic cause of impaired immune response in humans [1]. Infants are the most vulnerable part of the population, since the incidence of allergic and infectious diseases increases most during prepubertal age [2, 3].

Organochlorine pesticides exhibit a wide range of endocrine disrupting properties [4]. Pesticide dichlorodiphenyltrichloroethane (DDT) is an organochlorinated compound with explicit antithyroid and antiandrogenic properties [5, 6]. Recent studies demonstrated that DDT disrupts production of adrenal steroid hormones and catecholamines [7, 8]. Its extensive use in the 20th century and reintroduction of DDT in the 21st century into the vector disease control, supported by the World Health Organization, greatly contributed to the spread of DDT throughout the world and made its effects in small doses universal [9]. DDT readily crosses the placental barrier and is excreted in milk, so its possible effects on organ development are of great concern [10, 11]. We have previously demonstrated that developmental exposure to low doses of DDT resulted in changes in the rate of thymic growth and preservation of the functional maturation of thymic lymphocytes [12, 13]. Damage to the central organs of immunity by an endocrine disruptor can probably lead to dysfunction of lymphocytes in peripheral lymphoid organs. It is known that primed lymphocytes transform into blasts and proliferate after repeated administration of antigen. The in vitro transformation reaction of blasts in response to mitogens and the assessment of the proliferative response allows assessing the functional potential of lymphocytes [14, 15]. In the present study, we focused on assessing the proliferative response of splenic lymphocytes to the T cell mitogen concanavalin A and evaluating splenic morphology in prepubertal rats exposed to DDT during prenatal and postnatal development.

Material and Methods

Chemicals

We purchased *o,p'*-isomer of DDT from the Sigma-Aldrich (USA). An aqueous solution with 20 µg/L *o,p'*-DDT was prepared to replace tap water as a route of exposure in animals.

Laboratory animals and experimental design

Female and male Wistar rats were purchased from the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The rats were kept at a temperature of 22-23 °C and were fed with standard pelleted food without restrictions. The absence of DDT, its metabolites and related organochlorine compounds in standard pelleted food and tap water was confirmed by high-performance liquid chromatography and mass spectrometry data obtained at the Moscow Center for Hygiene and Epidemiology.

Female rats (n=4) received a solution of *o,p*-DDT (20 µg/L) instead of tap water from the moment of mating during their pregnancy and lactation. The rats had free access to the DDT solution. The average daily intake of DDT, calculated as the average daily volume of consumed DDT solution per kg of body weight, was 2.51 ± 0.12 µg for pregnant females and 2.65 ± 0.11 µg for lactating females. These exposure levels meet the criteria for exposure to low doses of DDT [9]. Only male offspring (n=16) were included in the study due to gender-based differences in growth rate and immune system histology in rats. The control group included male offspring of intact rat dams (n=14). Both rats exposed to DDT and control rats were sacrificed on postnatal day 7 with an overdose of Zoletil ® (Virbac Santé Animale, France). The spleens were surgically removed under sterile conditions. Rats and excised spleens were weighed. Relative spleen weight was calculated and presented as a percentage of body weight.

The study was carried out in accordance with the standards and rules for handling laboratory animals in compliance with the International Guiding Principles for Biomedical Research Involving Animals (1985), laboratory standards in the Russian Federation (Order of the Russian Federation Ministry of Healthcare No. 199n of April 1, 2016, On Approval of the Rules of Good Laboratory Practice). Animal experiments were approved by the Ethics Committee at the Institute of Human Morphology on October 27, 2021, protocol no. 28(4).

Splenocyte isolation

The spleen was homogenized in a 40-µm cell strainer and washed with RPMI 1640 cell culture medium supplemented with L-glutamine (2 mmol/mL), 10% heat-inactivated fetal bovine serum, and glutamine. After three cycles of centrifugation and washing, splenocytes were stained with trypan blue to assess viability. Cell viability always exceeded 95%. Cells were resuspended in RPMI 1640 Complete Medium (2×10^6 cells/mL) to proliferate *ex tempore* and to exhibit a proliferative response to mitogenic assays.

Ex tempore proliferation assay

Ex tempore proliferation assay is a method that allows evaluating the proliferative activity of cells, which is close to the actual rate of proliferation in vivo [16]. Cell suspension samples were transferred into a 96-well U-bottom plate in triplicate (0.1 mL per well). We then added 3H-thymidine (Sigma-Aldrich, USA) (1 µCi per well). The plates were incubated in an atmosphere with 5% of CO2 for 4 hours. Cells were collected onto glass fiber filter paper using a cell harvester. The uptake of 3H-thymidine by cells was assessed by filter counting using the 1209 Rackbeta liquid scintillation counter (LKB, Austria).

Splenic lymphocyte response to the mitogen

Splenocytes (105/200 mL) were incubated in triplicate in RPMI 1640 Complete Medium with 1 mg/mL of concanavalin A (Sigma, USA) or without stimulus in 96-well plates for 72 hours at 37 °C and 5% CO2 atmosphere in humidified air. Proliferating cells were identified by bromodeoxyuridine incorporation enzyme-linked immunosorbent assay using the Biotrak Cell Proliferation System (Amersham Biosciences, Freiburg, Germany). Optical density was measured at 450 nm. The analysis was carried out in accordance with the manufacturer's recommendations. The proliferative response to mitogen was determined using triplicate as follows:

Proliferation Index = Optical density of samples with concanavalin A / Optical density of samples without concanavalin A.

Histological examination and histomorphometry

Spleens were fixed in Bouin solution and, after standard histological processing, embedded in paraffin. Histological sections were stained with hematoxylin and eosin. The total surface area of the white pulp, the marginal zone area, and the number of hematopoietic cells per 1 mm2 of the red pulp were measured using Image Scope software (Leica Microsystems Gmbh, Germany).

Flow cytometry

The freshly obtained splenocyte suspension was washed twice by centrifugation in RPMI1640 for 5 min at 1,000 rpm and brought to a concentration of 10 million cells in 1 mL. The sample preparation procedure was carried out according to standard protocols. The percentage of T cells was assessed by flow cytometry using fluorochrome-conjugated antibodies to CD3 (eBioscience, USA). FC500 flow cytometer (Beckman Coulter, Germany) was used in this study.

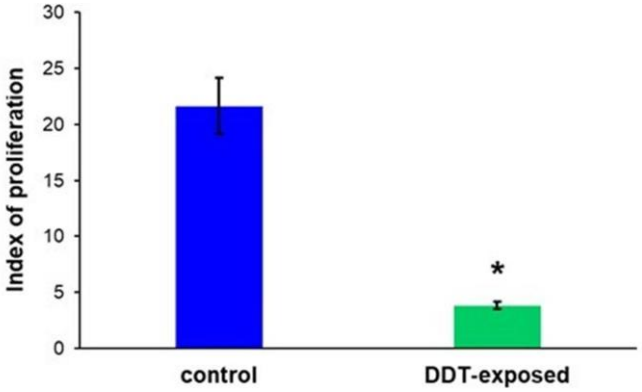

Figure 1. Proliferative response of splenic lymphocytes to mitogen concanavalin A in the 7-day-old rat pups: control (blue) and developmentally exposed to low doses of DDT (green).

* p<0.05 compared to the control.

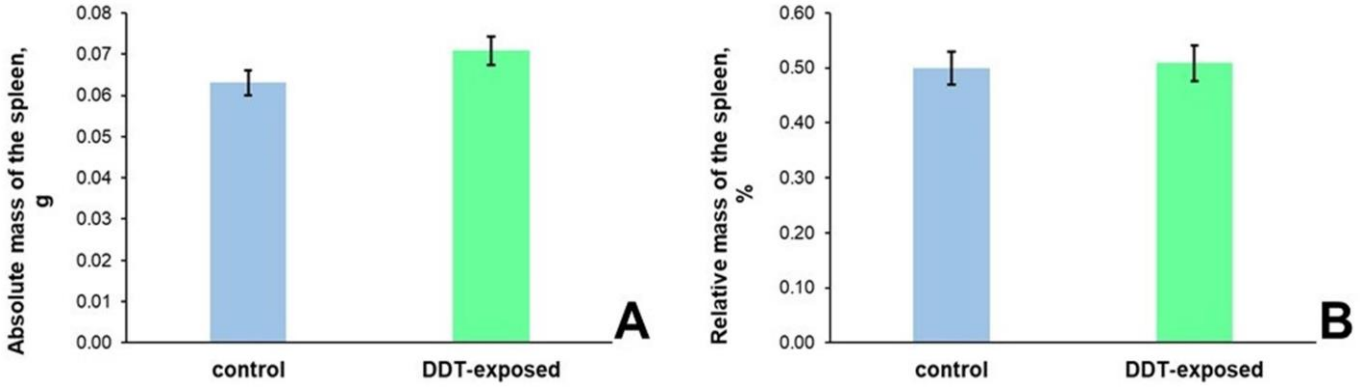

Figure 2. Absolute mass and relative mass of the spleen in the 7-day-old rat pups: control (blue) and developmentally exposed to low doses of DDT (green), M±SD.

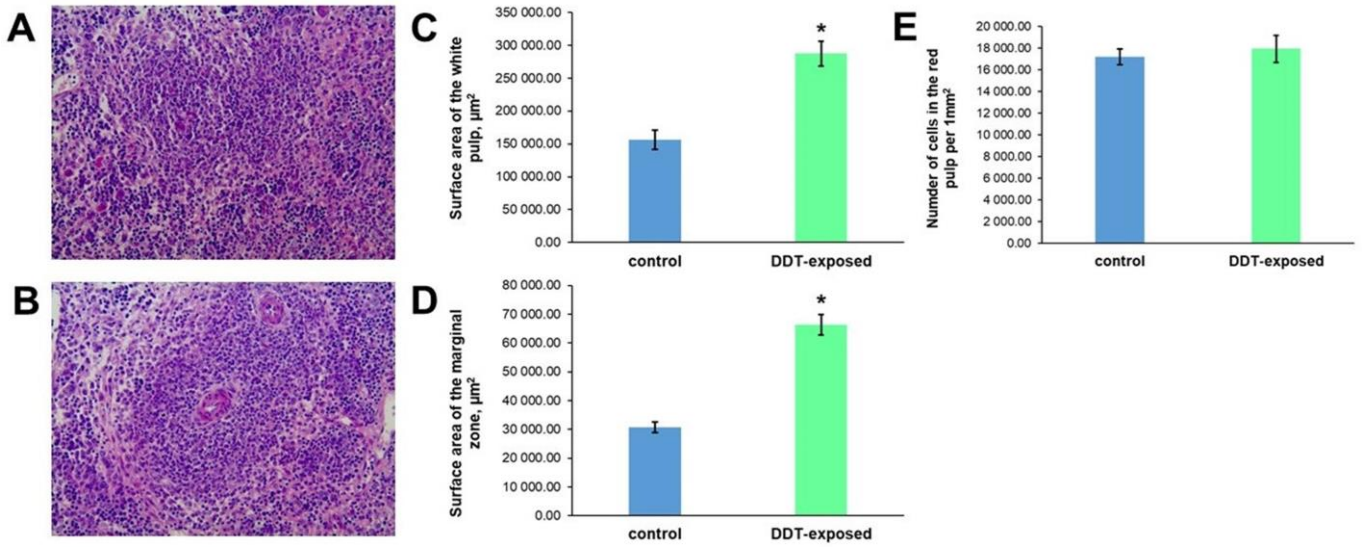

Figure 3. Histological parameters of the spleen in the 7-day-old rat pups: control (blue) and developmentally exposed to low doses of DDT (green).

Typical structure of the periarteriolar lymphoid sheath of the control (A) and DDT-exposed (B) rats, hematoxylin and eosin staining, magnification ×200; (C) the surface area of the white pulp in the spleen; (D) the surface area of the marginal zone in the spleen; (E) the number of hematopoietic cells in the red pulp of the spleen, M±SD. * p<0.05 compared to the control.

Statistical data processing

The data were processed using STATISTICA 7.0 software (StatSoft, Inc., USA). Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk tests were employed to assess the normality of distributions. Central tendencies and the variance of the quantitative characters with an approximately normal distribution were described using the mean and standard deviation (M±SD). Comparison of the groups was performed using the Student's *t*-test and χ^2-test. The differences were assumed statistically significant at p<0.05.

Results

Proliferative response of splenic T cells to concanavalin A

The control rats demonstrated a pronounced proliferative response of splenic lymphocytes to the mitogen concanavalin A. Rats exposed to DDT exhibited significantly reduced index of proliferation (*Figure* 1).

Morphology of the spleen

Morphological evaluation revealed no differences in the spleen anatomy between the control and DDT-exposed rats. Relative weights of the spleen were also similar (*Figure* 2).

Histological examination revealed some differences in the structure of the spleen. In the control rats, the white pulp represented periarteriolar lymphoid sheaths with developing marginal zone (*Figure* 3A, C). The marginal zone was observed in approximately a quarter of lymphoid sheaths. Its share in the white pulp composition was small (*Figure* 3D). The marginal zone was represented mainly by mononuclear cells and a few granulocytes. The red pulp was represented by reticular tissue with abundant hematopoietic cells (*Figure* 3E).

In the DDT-exposed rats, the splenic parenchyma was represented by forming periarteriolar lymphoid sheaths (*Figure* 3B). The surface area of the white pulp was larger than in control animals (*Figure* 3C). Marginal zone was detected in more than a half of lymphoid sheaths, and its size also exceeded the values in the control group (*Figure* 3B, D). Red pulp contained a similar number of hematopoietic cells (*Figure* 3E).

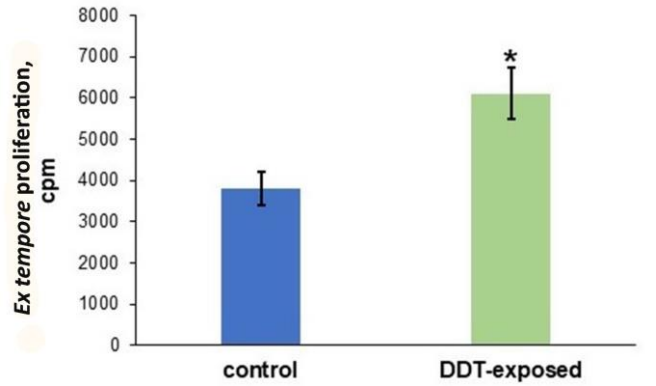

Figure 4. *Ex tempore* proliferation rate of splenocytes in the 7-day-old rat pups: control (blue) and developmentally exposed to low doses of DDT (green), M±SD.

* p<0.05 compared to the control.

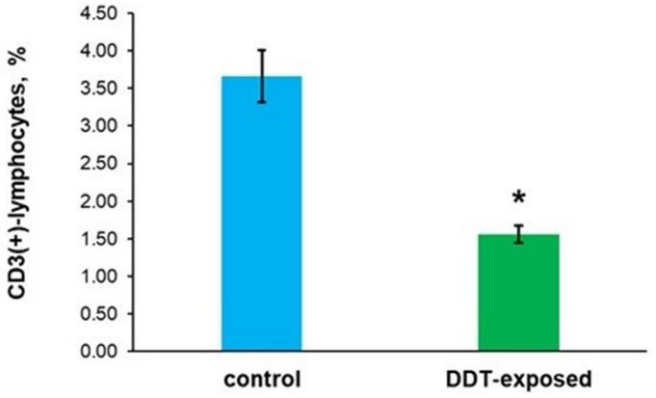

Figure 5. Share of CD3 positive lymphocytes in the spleen of the 7-day-old rat pups: control (blue) and developmentally exposed to low doses of DDT (green), M±SD.

* p<0.05 compared to the control.

Proliferation rate of splenocytes

Quantification of *ex tempore* proliferation resulted in significantly elevated rates of mitotic division in the splenocytes of DDT-exposed rats (*Figure 4*).

Quantification of T cells

Flow cytometry revealed differentiated CD3 positive lymphocytes in the spleens of control and exposed rats, but the share of T cells in DDT-exposed animals was half as high (*Figure 5*).

Discussion

It is known that the functional maturation and structural development of the immune system are extremely sensitive to external influences. It has been shown that prenatal exposure to such influences not only affects morphogenetic processes in the central and peripheral organs of immunity, but also disconnects epese processes [12, 16]. The spleen is the largest peripheral organ of immune defense, which in ontogenesis is exposed both to the influence of the central organs of the immune system and to the impact of various factors that directly interfere with the development of lymphoid formations and stromal components.

Studies have shown that splenectomy in humans and rodents significantly affects the immune response to bacterial and viral infections and alters antitumor immunity [17, 18]. In both humans and rats, a protective type of spleen is observed [19]. In rats, the formation of lymphoid structures of the spleen occurs in postnatal development, which makes it a convenient model for studying the effects of various factors on morphogenetic processes. The functional activity of spleen lymphocytes is traditionally assessed by the blast transformation reaction induced by exposure to a mitogen, since only differentiated mature lymphocytes are capable of transforming into blasts with their subsequent proliferation under the influence of antigens. In our study, we used concanavalin A, which is the most potent activator of T lymphocytes, including NKT cells. These populations of CD3-expressing lymphocytes (conventional T cells and NKT cells) are part of the innate immune system and are among the first to respond to pathogens. They also play an important role in antitumor immunity [20].

In rats exposed to the endocrine disruptor DDT, we observed a reduced prepubertal T cell proliferative response. Analysis of morphological data revealed that the lymphoid formations of the spleen develop at an accelerated pace, as evidenced by a large proportion of white pulp, better developed marginal zones and an increased rate of cell proliferation. It is well known that only highly differentiated and nondividing lymphocytes are capable of responding to mitogen stimulation with blast transformation. Flow cytometry revealed a significantly lower percentage of CD3 positive cells in the spleen of rats exposed to DDT. Clearly, the lower content of differentiated thymic T cells was responsible for the lower proliferative response in this case.

Since the mitogen-induced blast transformation reaction reflects the functional maturity of lymphocytes, our data indicate an impairment of cellular immune responses in the prepubertal period in rats. These responses developed under the influence of low doses of DDT. It is important to note that the accelerated morphological development of the white pulp in the spleen of exposed animals is not an indicator of its higher functional development. Hence, our findings suggest that low-dose exposure to the endocrine-disrupting chemical DDT should be considered a risk factor for the maturation of the immune response.

Conclusion

Developmental exposure to low doses of the endocrine disrupter DDT impairs functional and morphologic maturation of the spleen in prepubertal rats. DDT accelerates formation of lymphoid compartments and reduces functional maturation of the spleen as the organ with an immune function. Reduced rates of proliferative response in splenic T cells imply delayed immigration of thymic T cells to the spleen.

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Conflict of interest

The authors declare no conflicts of interest.

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Authors:

Bella B. Gagulaeva – PhD student, Laboratory of Endocrine System Development, Avtsyn Research Institute of Human Morphology of Petrovsky National Research Center of Surgery, Moscow, Russia. http://orcid.org/0000-0002-1415-3070.

Nataliya V. Yaglova – MD, DSc, Head of the Laboratory of Endocrine System Development, Avtsyn Research Institute of Human Morphology of Petrovsky National Research Center of Surgery, Moscow, Russia. http://orcid.org/0000-0002-8239-5039.

Sergey S. Obernikhin – MD, DSc, Senior Researcher, Laboratory of Endocrine System Development, Avtsyn Research Institute of Human Morphology of Petrovsky National Research Center of Surgery, Moscow, Russia. http://orcid.org/0000-0003-0997-6766.

Ekaterina P. Timokhina – PhD, Senior Researcher, Laboratory of Endocrine System Development, Avtsyn Research Institute of Human Morphology of Petrovsky National Research Center of Surgery, Moscow, Russia. http://orcid.org/0000-0003-0504-1488.