A new experimental model of hemolytic anemia after butoxyethanol and the study of its immunology

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Abstract
2-butoxyethanol (C₆H₁₄O₂) is widely used in many industrial reagents; according to the in vitro data it was established that 2-butoxyethanol metabolites are strong haemolytic poisons. Ghanayem B.I., Sullivan Ch.A. (1993) investigated in vitro the effect of BE on the red blood cells of 10 species of mammals, including humans. In this study, the authors established the species specificity with regard to the development of hemolytic anemia under the effect of butoxyethanol [4]. In the context of the available data, creation of experimental model based on the introduction of animal butoxyethanol is taking place. Since the drug-induced hemolytic anemia is formed at the adjacency of toxic and autoimmune forms, the study of immunochemistry of any toxic anemia is of great interest. Objective: to develop a new experimental model of hemolytic anemia after butoxyethanol and to study its immunology. In conclusion, the proposed model of hemolytic anemia after butoxyethanol may be used in the experimental and preclinical studies. The intraperitoneal administration of butoxyethanol provokes an autoimmune response directed against own red blood cells. The intraperitoneal administration of butoxyethanol to experimental animals is accompanied by a reduction of the lymphoid tissue that corresponds to the appropriate response to stress in the central and peripheral organs of immunogenesis.

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Introduction

2-butoxyethanol (C₆H₁₄O₂) is widely used in many industrial reagents; according to the in vitro data it was established that 2-butoxyethanol metabolites are strong haemolytic poisons. Ghanayem B.I., Sullivan Ch.A. (1993) investigated in vitro the effect of BE on the red blood cells of 10 species of mammals, including humans. In this study, the authors established the species specificity with regard to the development of hemolytic anemia under the effect of butoxyethanol [4]. In the context of the available data, creation of experimental model based on the introduction of animal butoxyethanol is taking place.

Since the drug-induced hemolytic anemia is formed at the adjacency of toxic and autoimmune forms, the study of immunology of any toxic anemia is of great interest.

Objective: to develop a new experimental model of hemolytic anemia after butoxyethanol and to study its immunology.

Materials and methods

The experiment involved 40 animals - nonlinear white rats (males and females), four months of age, weighing 150-200g which were kept under standard conditions of experimental and biological clinics-vivarium (free access to food and water, and 12-14h daylight). Experiments were carried out in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" Strasbourg, 18.III.1986 (Text was amended according to the provisions of the Protocol (ETS No. 170) after of its entry into force on 2 December 2005; The Treaty of Lisbon amending the Treaty on European Union and the Treaty establishing the European Community entered into force on 1 December 2009) at the Central Research Laboratory of the State Budgetary Institution of Higher Professional Education "Perm State Academy of Medicine named after Academician E. A. Wagner" of Ministry of Health of the Russian Federation.

Laboratory animals were injected intraperitoneally once 2-butoxyethanol (BE) in an empirically chosen dosage of 20mg/kg body weight (4mg per animal). The working solution was prepared as follows: 180mcL of pure BE (1ml of 1g substance), diluted with 45mL of water for injection. As a result, 1mL of the working solution contained 4mg of BE (dosage per animal). Intraperitoneally 1mL of the working solution was injected. Two experimental groups of 20 animals...
each were formed: group I - intact animals in the standard vivarium conditions, group II - the introduction of BE. Daily observation of the animals included registration of behavior, appearance, physiological functions. Prior to the experiment, and upon its completion, we examined the following animal peripheral blood indices: number of red blood cells, reticulocytes (was determined as a percentage); hemoglobin concentration. Before starting the experiment, animal venous blood was taken in the amount of 60-100mcL for the individual erythrocyte suspension and serum samples. On day 10 of the experiment, the animals were withdrawn from the experiment under ether anesthesia and euthanasia according to the above rules. The autopsy was performed for further histological studies (spleen, thymus, Peyer's patches), and the venous blood sampling for serum agglutination tests.

The histological study was carried out in accordance with standard methods using hematoxylin and eosin staining [7]. Visualization and photography of micropreparations was performed on a microscope Micros MC 50 (Austria) in the program Scope Photo (Camera CAM V200). Quantitative image analysis was performed in the program Image J (BioVision, Version 4.0). The histometric analysis of the spleen tissue was performed using the function "the measurement of the linear objects."

After the experiment, the agglutination of own red blood cells (obtained at the beginning of the experiment) with animal serum was carried out. In agglutination, serum was dissolved with a double step in the range of 1:2 to 1:2048 (11 holes) in 96-well plastic plates for immunological reactions (*MEDPOLIMER* Sankt Petersburg) in isotonic sodium chloride solution supplemented with 0.2% human serum albumin (pH=7.3) in a volume of 25mcL per each hole of microtiter plate. Then 25mcL of a 0.5% suspension of the erythrocytes which was prepared immediately before the reaction were added to each hole of the plate. Samples were incubated for 2h at 4°C, and then for 30min at a temperature of 37°C (to detect the action of heat and cold antibodies). The results of the reaction were evaluated by a conventional method, and expressed with the log-normal distribution of data in the form of inverse log₂-titer [6]. The results of the reaction were re-evaluated after 24h of storage at 4°C. Statistical analysis of the data was made using the software BioStat 2008. When characterizing the samples, the sample mean, standard error and standard deviation were calculated; comparison of the two samples was performed using Mann-Whitney test and Wilcoxon test. The critical level of significance difference was considered P<0,05.

**Results**

In the experiment, no cases of death of the animals were marked. In the analysis of peripheral blood of the experimental group by the end of experiment there was a significant decrease in hemoglobin concentration and reduction in the number of erythrocytes; in the group of intact animals the changes in the red blood cells were not observed during the experiment (Table 1). The results of the study of reticulocyte number in smears were the following: intact animals: 1,429±0,192% before the experiment, 1,787±0,235% at the end of the experiment; (P>0,05); introduction of BE: 1,488±0,115% before the experiment, 4,322±0,127% at the end of the experiment (P<0,05). The results of agglutination: group I-no agglutination in all holes of the plate; group II - agglutination test was positive in all animals (100%); average values log - 6.7±0.15. Thus, the presence of antibodies against their own erythrocytes in the experienced group showed that the introduction of butoxyethanol causes the development of an autoimmune response directed against their own red blood cells.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of erythrocytes before the experiment</th>
<th>Number of erythrocytes at the end of the experiment</th>
<th>Hb level before the experiment</th>
<th>Hb level at the end of the experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I, n=20</td>
<td>6,456±0,57</td>
<td>6,532±1,88</td>
<td>118,4±9,82</td>
<td>122,8±5,57</td>
</tr>
<tr>
<td>Group II, n=20</td>
<td>6,342±0,88</td>
<td>4,556±0,15*</td>
<td>122,4±3,544</td>
<td>84,0±5,177*</td>
</tr>
</tbody>
</table>

P<0,05 - with respect to the original data; the method of statistical analysis - Mann-Whitney test

The histological structure of thymus of group 1 corresponded to the species norm. The thymus was covered with a connective tissue capsule and divided by partitions into clearly demarcated segments, consisting of cortical and medullary layers. Few thymic cells were detected in the medullary layer. Histological examination of the thymus tissue of animals of group II revealed the availability of the plots of lymphocyte death in cortical and medullary layers. At the same time, in 40% of the animals the death of lymphoid cells in the cortical layer was more pronounced than in the medullar layer, allowing the existence of the phenomenon of inversion of the layers; 40% of the animals had the disappearance of the inversion layer. However, in 20% of the animals of this experimental group the structure of the lobules was not violated. In four animals of this group (20%) the lymphoid tissue in the lobules was replacement by adipose tissue. Also in all the animals the collagenization of stroma was revealed in the cortical and medullary layers. In four animals of this group (20%) the cystic advanced thymic cells were detected in the medulla of the thymus. In 70% of the animals the epithelial tubules lined by cubic epithelial cells were also detected in slices of the cortical layer (Fig. 1). In all animals treated with intraperitoneal administration of BE, mast cells identified in a large amount were detected in the thymus tissue. These are large cells with basophilic granules often have an irregular shape (Fig. 2). Mast cells were found mainly in the interlobular connective tissue, in the connective tissue near the walls of blood vessels and also in thymic...
parenchyma - in the cortex slices in vast plots of lymphoid death. Thus, in animals with intraperitoneally administered BE for the purpose of simulating toxic hemolytic anemia, we detected the symptoms of accidental involution of the thymus, expressed in varying degrees and corresponding to different stages of involution accompanying the acute stress (by Hans Selye).

The structure of the spleen of intact animals corresponded to the species norm: the red and white pulps were detected in the tissue. The white pulp is formed by spherical lymphoid formations (splenic lymphoid nodules) with light centers (B-dependent area). The central artery, surrounded by a periarterial collar, is seen well in the node (T-dependent area). Splenic septa are thin, they contain arteries and veins. The relative volume of white splenic pulp of healthy rats is 30.18±2.69%. In the spleen of animals of group II a significant decrease in the relative volume of white pulp (16.33±0.55%; P<0.05 compared with the control group) was detected. Lymphoid nodules have a typical structure. All animals have hemosiderosis of the red pulp (100%), depletion of the cellular elements of the red pulp (100%), sinusoidal congestion (80%). In the red and white pulps the regions of destruction of lymphoid cells were identified. Thus, the study of animal spleen exposed to BE administration showed the same pattern as in the study of thymus tissue: the reduction of lymphoid tissue corresponding to the body response to stress.

The changes in thymic lymphoid tissue of animal treated with BE are very interesting, in particular, the appearance of a large number of epithelial tubules and mast cells. It is known that the epithelial tubules occupy a special place among

Discussion

In the experimental animals intraperitoneally injected BE, the development of hemolytic anemia was observed because of decrease in erythrocyte number and hemoglobin concentration, and increase in reticulocyte amount which was detected in the animals' peripheral blood. The presence of antibodies against their own erythrocytes in the experimental group showed that introduction of butoxyethanol also causes the development of an autoimmune hemolytic response. This can be explained by the fact that the drug-induced hemolytic anemia is formed at the adjacency of toxic and autoimmune forms; and as we know that many chemicals and drugs can act as haptons.

We found the reduction of lymphoid tissue of the thymus, spleen and Peyer's patches in experimental group of animals. These changes are similar to those in the lymphoid tissue that occur during stress. There are two groups of factors that can cause significant stress-induced immune suppression: direct activation of neuroendocrine axis as a result of increased production of hypothalamic corticotropin-releasing hormone (CRH)-this group includes the effects on toxic substances and emotional stress; indirect activation of the neuroendocrine axis-in infectious diseases and unbalanced diet [5]. That is, the resulting acute poisoning changes in the immune system are considered as similar to those that occur in response to stress [11].

The changes in thymic lymphoid tissue of animal treated with BE are very interesting, in particular, the appearance of a large number of epithelial tubules and mast cells. It is known that the epithelial tubules occupy a special place among
the thymic epithelial structures; they occur in human thymus and all kinds of animals in the early postnatal period; within the lobules the epithelial tubules selectively localized at the cortico-medullary border, unlike thymus cells which normally are localized exclusively in the thymic medulla layer. In the literature there are described changes in the morphology of the epithelial tubules in the thymus of different species of animals under the influence of stress on the body, endocrine factors, and the antigens; these changes are regarded as compensatory-adaptive reorganization of the epithelium [10]. The mast cells are an integral part of the thymic microenvironment; they are regulators of the tissue homeostasis and are involved in the general adaptation reaction at a cellular level. Mast cells accumulate the hyaluronic acid in the extracellular matrix and optimize the migration of lymphocytes [10]. The role of mast cells in the body refers to the number of unsolved problems. Now it is known that the implementation of immune response requires cooperation of lymphoid (T- and B-lymphocytes) and non-lymphoid cells - macrophages and mast cells [3]. Mast cells contribute significantly to the activation of the death process of thymic lymphocytes under stress which is accompanied by their degranulation [8].

In conclusion, the proposed model of hemolytic anemia after butoxyethanol may be used in the experimental and preclinical studies. The intraperitoneal administration of butoxyethanol provokes an autoimmune response directed against own red blood cells. The intraperitoneal administration of butoxyethanol to experimental animals is accompanied by a reduction of the lymphoid tissue that corresponds to the appropriate response to stress in the central and peripheral organs of immunogenesis.

The authors declare that they have no conflicts of interest.

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