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PECULARIRTIES OF INNATE IMMUNITY RECEPTORS EXPRESSION BY LYMPHOCYTES OF MESENTERIC LYMPH NODES IN OFFSPRING OF EXPERIMENTAL GESTATIONAL DIABETES RAT

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Abstract

The purpose of the research was to find out the distribution of TLR2+-, TLR4+-, NOD2+- and RIGI+-lymphocytes in mesenteric lymph nodes in the offspring of experimental gestational diabetes (EGD) rats, in the offspring of rats with EGD whose mothers received glibenclamide and the descendants of rats from EGD who received oral insulin for 14 days after birth.

Materials and methods. For the identification of PRR+-lymphocytes was used an indirect immunofluorescence method using monoclonal or polyclonal antibodies.

Results. Experimental gestational diabetes leads to an increase in the number of TLR2+, TLR4+, NOD2+ and RIGI+ lymphocytes in MLN in the offspring and changes the density of PRRs on immune cells. Changes are more pronouncedly in age 1 month of life. In conditions of formation of oral tolerance to insulin, the number of cortex TLR2+ and TLR4+ lymphocytes in 1-month offspring decreases. The amount of TLR2+ and RIG-I+ cells in medullary cords of these rats decreases too. The dynamics in the number of immunoreactive cells to PRRs in the cortex of the MLN remains up to 6 months of age. Administration of glibenclamide to pregnant females reduces the number of TLR4+ and RIG-I+ lymphocytes in the MLN of 1-month offspring, but only TLR2+ cells in 6-month-old rats. It does not affect their number in medullary cords and decrease preferentially the PRRs density on immunopositive MLN lymphocytes in early terms of observation.

Conclusions. The introduction of insulin to offspring and glibenclamide to pregnant females reduces the level of activation of the innate immunity receptors in the MLN in offspring.

Keywords: *pattern-recognizing receptors, mesenteric lymph nodes, innate immunity, experimental gestational diabetes.*

Introduction

Mesenteric lymph nodes (MLN) are a major site of induction of peripheral immunological tolerance (PIT) to a variety of antigens, including pancreatic [1, 2], and intranatal hyperglycemia, which develops in gestational diabetes (GD), can affect organ morphogenesis and leads to violations of the formation of PIT [3]. The use of mucous membranes is an attractive route for the introduction of antigens as tolerogens, and in the offspring of rats with experimental gestational diabetes (EGD) there are significant violations of PIT formation in MLN: repression of the AIRE gene, decreased mRNA Deaf1, transcription factor Foxp3, T Treg) [4]. Oral administration of insulin in the first 2 weeks of life eliminates these changes, but the mechanisms of this effect need further clarification. It is known that the activation of the adaptive immune response, in particular the differentiation of the Treg subpopulation, is impossible without prior signaling from the receptor components of innate, namely a number of membrane toll-like receptors (TLR), cytoplasmic Nod- (NLR) and RIG-I-like receptors RLR). These receptors after activation induce the synthesis and secretion of cytokines and costimulatory molecules through transcription factors of the NF-κβ family [5].

In addition, an important part of the pathogenesis of EGD is the activation of one of the NOD-like receptors of innate immunity (RII) - NLRP3-inflamasoma [6, 7]. Among the inhibitors of inflamasoma is promising glibenclamide, which can also effectively correct hyperglycemia in pregnant women [8]. We have previously found that the development of EGD is accompanied by transcriptional induction of the Nlrp3 gene in MLN in offspring [9], increases the number of NLRP3 + lymphocytes, and glibenclamide, as an inhibitor of NLRP3 activation, has been shown to be effective only in the early stages [10]. There are no data on the effects of glibenclamide on other RIIs at all.

Methods

The studies were performed on female Wistar rats obtained from the nursery of the Association of Veterinary Medicine PE "Biomodelservice" (Kiev). The studied animals were divided into 8 experimental groups of 20 individuals: the offspring of intact Wistar rats (males) aged 1 month (group 1) and 6 months (group 2), which on the 15th day of the dated pregnancy was injected once / alternately 0, 5 ml of 0.1 M citrate buffer (pH = 4.5); offspring of Wistar rats (males) with experimental gestational diabetes (EGD) aged 1 month (group 3) and 6 months (group 4), which on the 15th day of the dated pregnancy was injected once / alternately with streptozotocin at a dose of 45 mg / kg; Descendants of 1-month-old EGD rats (group 5) who were orally pipetted short-acting human insulin (ACTRAPID® HM, NOVO NORDISK, Denmark) for the first 14 days of life at a dose of 30 IU (1050 μg = 1.05 mg, 1 MO corresponds to 35 μg of anhydrous human insulin); offspring of rats with EGD at the age of 6 months (group 6), which were orally administered insulin at a dose of 30 IU for the first 14 days of life; offspring of rats with EGD at the age of 1 month (group 7) and 6 months (group 8), which from the 15th day of the dated pregnancy, along with a single intraperitoneal injection of streptozotocin at a dose of 45 mg / kg for 7 days orally intragastrically (in / w) injected glibenclamide (Farmak, Ukraine) at a dose of 5 mg / kg.

To identify the distribution of $TLR2^+$ -, $TLR4^+$ -, $NOD2^+$ - and $RIGI^+$ lymphocytes in PLN, an indirect immunofluorescence method was used using monoclonal or polyclonal antibodies to the corresponding PRR (Santa Cruz Biotechnology, USA). The population structure of immunopositive cells was studied on the basis of analysis of serial histological sections and data of their morphometric and densitometric characteristics. For this analysis we used a rotary microtome MICROM HR-360 (Microm, Germany), a Primo Star microscope (ZEISS, Germany), a highly sensitive camera AxioCam 5c (ZEISS, Germany), a software package for obtaining, archiving and preparing images for publication AxioVision 4.7. 2 (ZEISS, Germany) and computer program Image J (NIH, USA). Receptor density was determined taking into account the fluorescence intensity of the identified immunopositive cells and non-specific fluorescence of the drug (so-called "background"). On the basis of these indicators the corrected cellular fluorescence (in conventional units of fluorescence intensity UOIF) was calculated. This allowed us to calculate the absolute (number of cells per 1 mm^2 of tissue area) and relative (%) density of distribution of immunopositive

lymphocytes of different classes in the studied areas of PLN. Immunopositive cells located in the cortical plateau (CP) and muscle fibers (MF) of MLN were researched.

All experimental data were processed on a personal computer with the STATISTICA 6.0 package (Stat-Soft, 2001). The values of the arithmetic mean (M), its variance and the error of the mean (m) were calculated for all indicators. To determine the significance of differences in the results of studies in experimental and control groups of animals, the Student's ratio (t) was determined, after which the possibility of sample difference (p) and the confidence interval of the mean were determined. The critical level of significance in testing statistical hypotheses was taken to be 0.05.

Results

In the offspring of animals with EGD, there was an increase in the number of cells immunopositive to pattern-recognizing receptors in MLN. At the age of one month in the cortical plateau and cerebral cords, the total density of cells expressing all the studied PRR was significantly increased: in the cortical plateau TLR2 by 42% ($p < 0.05$), TLR4 2.9 times ($p < 0.05$) , NOD2 at 93% ($p < 0.05$), RIG-I 2.3 times ($p < 0.05$); and in the cerebral cords TLR2 by 76% ($p < 0.05$), TLR4 by 72% ($p < 0.05$), NOD2 by 2.2 times ($p < 0.05$), RIG-I by 39% <0.05) (Fig. 1 AB). It is noteworthy that in the cortical plateau membrane sensors of bacterial lipopolysaccharides TLR4 and cytoplasmic sensors of viral RNA RIG-I are more strongly activated, and in cerebral cords - membrane TLR2 and cytoplasmic NOD2 (See Fig. 1). only a significant portion of microbial ligands, but also damage-associated molecular patterns (DAMPs). The role of the latter can be not only classical damage factors, such as heat shock proteins and intercellular matrix, but also various metabolites, such as ATP, purines, fatty acids, changes in levels as observed in the conditions of EGD.

Analysis of PRR density on immunopositive lymphocytes in 1-month-old offspring of rats with EGD showed no significant changes in TLR4+ - and NOD2+ -cells, while the density of TLR2 in CP of this age group decreased by 11% ($p < 0.05$) in TLR2+ - limphoblast and 8% ($p < 0.05$) in TLR2+ - small lymphocytes. In MT of 1-month-old animals, the density of TLR2 in TLR2+ -lymphoblasts increased by 48% ($p < 0.05$), and in TLR2+ -small lymphocytes decreased by 13% ($p < 0.05$).

The study of lymphocytes' distribution in MLN expressing RII at the age of 6 months showed less significant changes. In particular, the total number of TLR2+ - (76%) and TLR4+ cells (2.1 times) increased significantly in the MLN CP, and only RIG-I+ lymphocytes (71%) in the cerebral cords (Fig. 1 C-D). Regarding the density of RII on immunopositive cells, it increased in TLR2+ -lymphoblasts of MF and decreased in TLR4+ -small and RIG-I+ -medium lymphocytes of MF, as well as in NOD2+ -medium and small lymphocytes of CP and MF.

The introduction of insulin affected the distribution of MLN cells expressing PRR as follows: at the age of 1 month in the cortical plateau there was a significant decrease in the total density of TLR2+ - by 31% ($p < 0,05$) and TLR4 + -lymphocytes by 46% ($p < 0, 05$), and in the cerebral cords TLR2+ - by 22% ($p < 0.05$) and RIG-I+ - by 20% ($p < 0.05$) (Fig.2 A-B). At the same time, the density of PRR on immunopositive lymphocytes in 1-month-old rats, which were orally administered insulin during the first 14 days of life, also mainly decreased, more clearly in CP. The dynamics of reducing the number of immunopositive cells to PRR in CP was maintained at 6 months.

In particular, the total number of TLR2+ lymphocytes was reduced by 37% ($p < 0.05$), $TLR4^+$ - by 41% and $NOD2^+$ - by 19% ($p < 0.05$). In brain strands, only the number of $NOD2^+$ cells decreased by 49% ($p < 0.05$) compared with animals with EGD (Fig. 2 C-D). The density of TLR2, TLR4 and NOD2 receptors in this age group decreased in young forms of immune cells - lymphoblasts, with the exception of RIG-I.

After administration of glibenclamide in one-month-old rats in the cortical plateau, a decrease in the number of $TLR4^+$ lymphocytes by 38% and $RIG\text{-}I^+$ - by 24% ($p < 0.05$) was observed compared with animals with EGD (see Fig.2 A). In 6-month-old rats, a decrease in the number of $TLR2^+$ cells alone by 35% was recorded in the CP of MLN (see Fig. 2 C). At the same time in cerebral cords significant changes did not occur at all (see Fig.2 B-D). Analysis of PRR density on immunopositive MLN lymphocytes after administration of glibenclamide showed a decrease

in this indicator in 1-month-old offspring of rats with EGD and divergent changes in 6-month-olds.

Discussion

Our results are consistent with other data on the ability of GD to have a significant impact not only on the immune processes in the mother, but also in the offspring [11]. Experiments [12] have shown the role of TLR4 in patients with gestational diabetes mellitus (GDM). TLR4 expression levels in maternal peripheral blood monocytes and serum TNF-α levels were increased in women with HD compared to healthy pregnant women. These results suggest that TLR4-mediated release of inflammatory cytokines may be a factor leading to elevated glucose levels in patients with HD. In addition, it can be argued that TLR4 is one of the links in the pathogenesis of GD. This is confirmed by the results of increasing the level of TLR2 and TLR4 mRNA in peripheral blood monocytes in women with HD, as one of the indicators of early metabolic disorders in the development of HD [13].

There are almost any data on changes in RII expression in offspring from HD mothers. In particular, Li Q. et al. (2016) demonstrated that the offspring of proinflammatory cytokine IL-1β in spleen cells are increased in the offspring of rats with EGD [14] and these effects are TLR4- and TLR2-dependent. The role of TLR receptors in the development of immune disorders in offspring is discussed in the work of Yanai S. et al. (2016) [15]. Their studies suggest that diabetes induces excessive proinflammatory activation in neonates through a TLR5- or TLR1/2-mediated innate immune response.

Regarding the increase in the number of $NOD2^+$ and $RIGI^+$ lymphocytes in MLN in the offspring of rats with EGD, this may indicate that not only membrane toll-like receptors, but also cytoplasmic sensors of the innate immune system may play an important role in the pathogenesis of immune disorders. Such excessive activation of RVI, in our opinion, can be caused by at least two factors:

-firstly, in the development of HD in humans and EGD in experimental animals, there are significant violations of the intestinal microbiome in both mother and offspring [16], which significantly changes the level of ligands for RDP [17];

- secondly, EGD is accompanied by changes in the metabolic profile in offspring, including changes in amino acid metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism and fatty acids, which can also be recognized by RII [18, 19].

On the other hand, MLN is the main site for the induction of oral tolerance (OT) among other lymphoid tissues [4, 20]. It is known that OT cannot be induced in mice deprived of MLN, but this does not affect animals in which Peyer's patches have been removed [21]. Our attempt to develop oral insulin tolerance shows that in 1-month-old offspring, the number of cells expressing RII in both CP and MF MLN decreases. At the same time, the dynamics of reducing the number of cells immunopositive to PRR in CP MLN persists until 6 months of age. These results are consistent with the data of Bonifacio E. et al. (2015), who conducted studies to assess the immune response in the context of oral insulin to children at genetic risk of developing diabetes mellitus1 [22].

Conclusions

1. Prenatal hyperglycemia leads to an increase in the number of $TLR2^+$ -, $TLR4^+$ -, $NOD2^+$ - and $RIGI^+$ lymphocytes in MLN in offspring, more clearly at 1 month of age, changes the density of PRR on immune cells.

2. In the conditions of formation of oral tolerance to insulin at 1-month-old offspring in KP MLN the number of $TLR2^+$ - and $TLR4^+$ -lymphocytes decreases, in MT - $TLR2^+$ - and RIG-I^+ - cells. The dynamics of reducing the number of cells immunopositive to RDP in KP MLN persists until 6 months of age, accompanied by a predominant decrease in membrane density and concentration of cytoplasmic RIIin both age groups, primarily in lymphoblasts.

3. Administration of glibenclamide to pregnant females reduces the number of $TLR4^+$ - and RIG-I^+ - lymphocytes in the CP of MLN of 1-month-old offspring, only $TLR2^+$ -cells in 6-month-old offspring, do not affect their number in MT at all, MLN in the early stages of observation.

Acknowledgments

The authors declare that there are no conflicts of interest.

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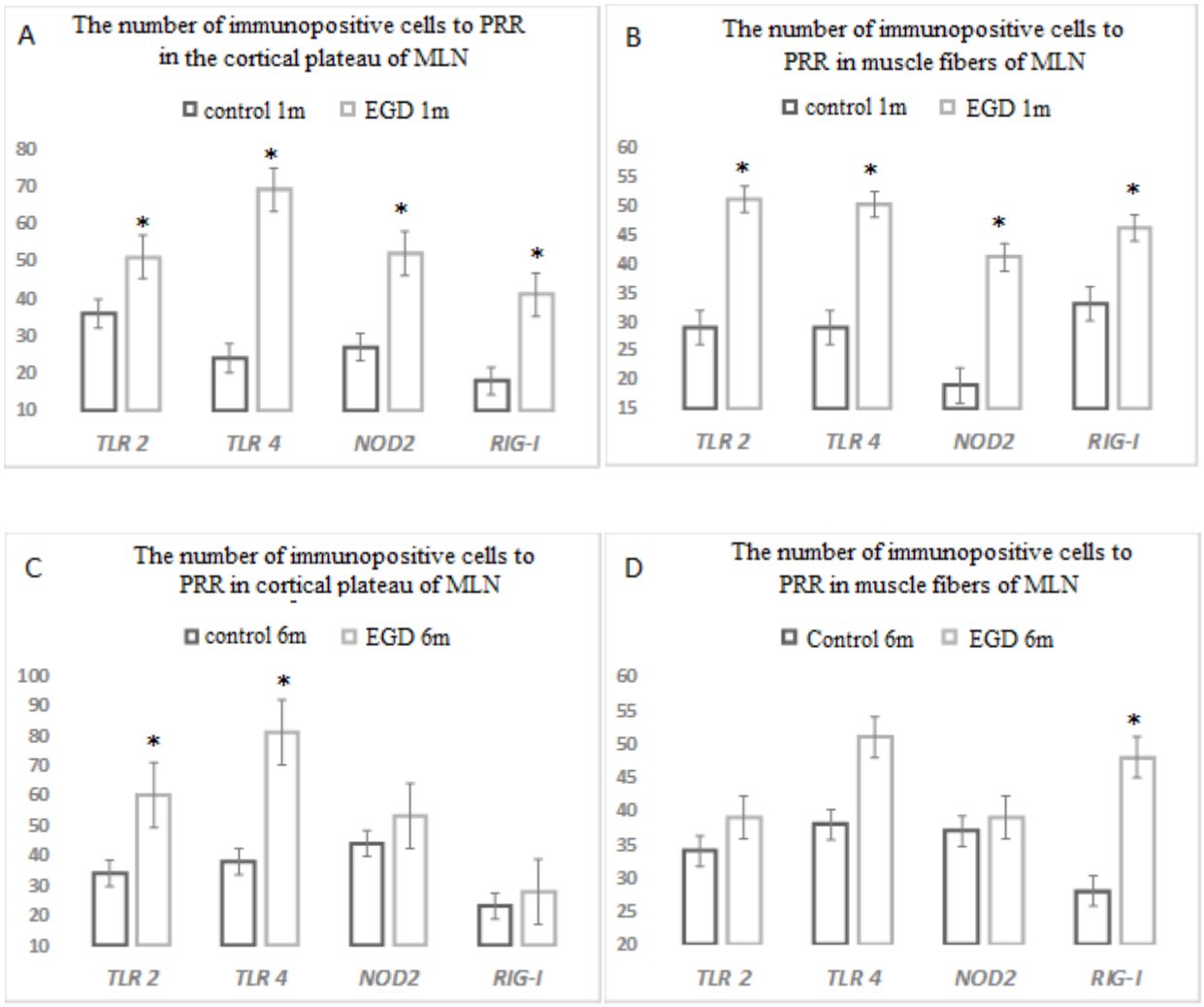
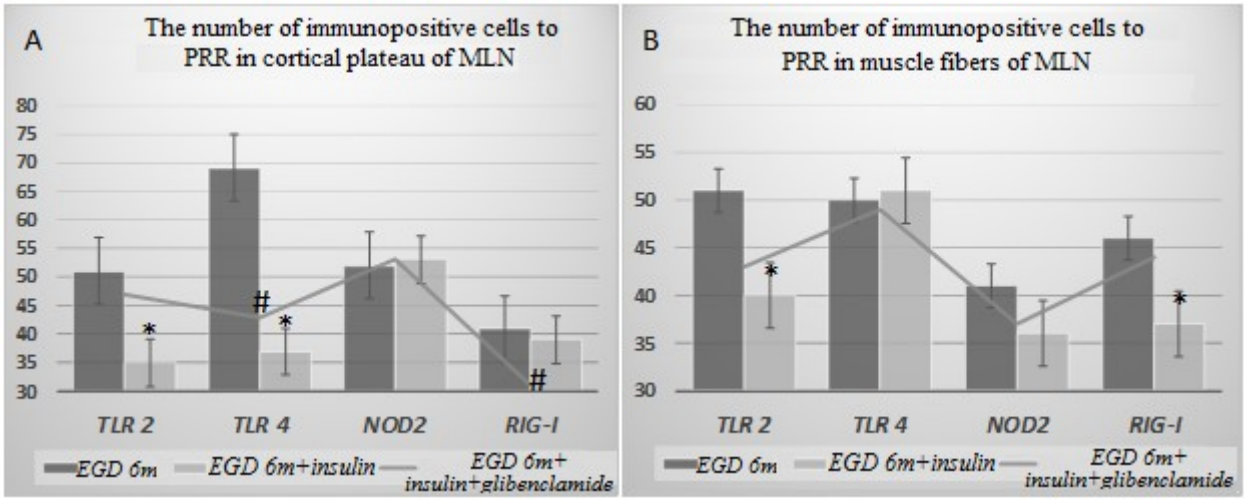


Figure 1. The number of $TLR2^+$-, $TLR4^+$ - $NOD2^+$ - and $RIG\text{-}I^+$ lymphocytes in MLN in the control group and in the offspring of rats with experimental gestational diabetes (EGD) at the age of 1 month (A, B) and 6 months (C, D). * - p <0.05. PRR - pattern-recognition receptors.



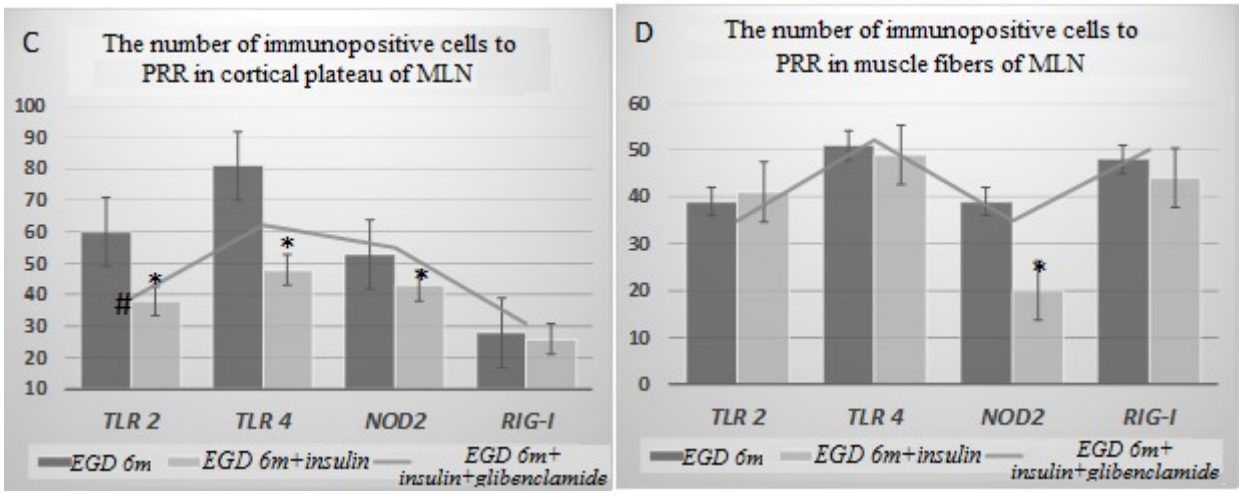


Figure 2. The number of $TLR2^+$ -, $TLR4^+$ - $NOD2^+$ - and RIG-I^+ lymphocytes in MLN of offspring of rats with experimental gestational diabetes (EGD) and after administration of glibenclamide to pregnant Wistar rats and rats receiving oral insulin for the first 14 days of life. At the age of 1 month (A, B) and 6 months (C, D).
* and # - $p < 0.05$