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Immune Proteasomes in the Developing Rat Thymus

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Abstract—The age dynamics of the content of the immune proteasome subunits LMP2 and LMP7 in rat thymus during prenatal and early postnatal ontogeny was studied. The LMP2 and LMP7 immune subunits were detected by Western blotting already by the 18th day of embryonic development, their amount increased to the 21st day to the level characteristic of the postnatal state. Double immunofluorescent labeling showed that in the thymus tissue the largest amount of LMP2 and LMP7 is localized in epithelial cells, whereas the level of their expression in thymocytes is lower. The results suggest that the establishment in thymus of selection processes, which depend on activity of immune proteasomes, can take place already in prenatal ontogeny. Analysis of age dynamics of the natural apoptosis level in thymocytes also favors this supposition. The presence of immune proteasomes in thymocytes during perinatal ontogeny suggests that, besides the antigen presentation, immunoproteasomes may possess other important functions.

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Multisubunit and multicatalytic proteinase complexes of eukaryotic cells known as proteasomes are involved in regulation of numerous cell processes, elimination of damaged proteins, and induction of immune response [1-5]. An important role in activation of T lymphocytes is played by immune proteasomes, among which those containing all catalytic subunits induced by γ-interferon are distinguished: LMP7, LMP2, and LMP10 (MECL-1), or LMP2 and LMP10 subunits, or proteasomes containing only LMP7 subunits [6]. Unlike all other proteolytic systems of cells, immune proteasomes most efficiently generate peptide fragments able to form complexes with major histocompatibility complex (MHC) class I molecules for presentation to CD8 T lymphocytes [7, 8]. In peripheral organs of the immune system, immune proteasomes are involved in presentation of foreign antigens to naive T lymphocytes, whereas in the central lymphoid organ, the thymus, they are responsible for negative selection of thymocytes [9, 10]. In antigen-presenting epithelial and dendritic thymus cells, immune proteasomes cleave autoantigens and use them for formation of antigenic epitopes that are exported to the cell surface in complexes with the class I MHC molecules for recognition and rejection of thymocyte clones carrying autospecific receptors [11, 12].

Recent data have shown that proteasomes of dendritic cells produce fragments of exogenous proteins and can also form antigenic peptides for presentation of the latter complexed with class II MHC molecules to naive $CD4^+$ T lymphocytes [13]. It is still not clear just what proteasomes, immune or constitutive, are involved in this process. However, these data suggest possible participation of proteasomes in negative selection of thymocytes both of CD8 and CD4 subpopulations.

Thymocytes undergo apoptosis during negative selection [14, 15]. However, mechanisms of autoantigen presentations in thymus are still poorly studied, and there are practically no data concerning the establishment of this process and the presence of immune proteasomes in prenatal and early postnatal ontogeny.

The goal of this work was to study the age dynamics both in the content of immune subunits LMP2 and LMP7 and in the level of natural apoptosis in thymus during perinatal ontogeny in rats.

Abbreviations: MHC) major histocompatibility complex; E18, E21) at 18 and 21 days of embryonic development; P1, P3, P5, P8, P19, P30) at 1, 3, 5, 8, 19, or 30 days of postnatal development.

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MATERIALS AND METHODS

Reagents. Polyclonal rabbit antibodies to proteasome immune subunits β1i (LMP2) and β5i (LMP7) from Affiniti (Great Britain), ECL kit, Hybond-ECL nitrocellulose membranes, and peroxidase-conjugated antibodies to rabbit IgG from Amersham Biosciences (Great Britain), monoclonal mouse antibodies to CD3 from BD Bioscience (Belgium), a mixture of mouse monoclonal antibodies to cytokeratins CK10, CK14, CK18, and CK19 from Novocastra (Great Britain), CY3-labeled second antibodies to rabbit IgG, FITC-labeled second antibodies to mouse IgG, PBS (0.1 M Na-phosphate buffer, 0.9% NaCl, pH 7.2-7.4, RNase A, and propidium iodide from Sigma (USA) were used in this work.

Animals. The work was carried out on Wistar rats: fetuses at the 18th (E18) and 21st (E21) day of embryonic development and at the 1st (P1) and following days of postnatal development (P3, P5, P8, P19, and P30). To obtain females with dated pregnancy term, three to four months old rats of 200-250 g were used. The day of spermatozoid detection in smear was considered as the first day of pregnancy. The day of delivery of newborn rats was taken as the first postnatal day. Pregnant females were kept under standard conditions with control illumination (from 5 to 19 h).

Preparation of cleared homogenates of thymus and spleen. Rat thymuses at different stages of embryonic and postnatal development were washed with PBS, blotted, weighed, and stored for several days at −70°C. Cleared homogenates were prepared at 0-4°C. Thymus portions of 100 mg were thawed and homogenized in microcentrifuge tubes (Costar, USA) with a Teflon plunger in 300 μl buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM $Na_2S_2O_5$, 1 mM PMSF, leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml). Homogenates were centrifuged for 30 min at 10,000*g*. Immune subunits of proteasomes were detected in supernatant (cleared homogenate) by Western blotting.

Western blotting. After SDS-PAGE of the cleared homogenates in 13% gel, the polypeptides were semi-dry transferred from the gel onto nitrocellulose membrane. The membrane was incubated for 2 h at 20°C in TNT buffer (10 mM Tris-HCl, pH 7,5, 150 mM NaCl, 0.1% Tween-20), then for 1 h in TNT buffer containing 5% dry defatted milk and polyclonal antibodies to immune subunits β1i (LMP2) or β5i (LMP7) in a dilution 1 : 2500. Then the membrane was washed several times with TNT buffer and incubated for 1 h in TNT buffer containing 5% dry defatted milk and the peroxidase-conjugated antibodies to rabbit IgG at dilution 1 : 5000. After washing in TNT buffer, the membrane underwent standard treatment with the ECL system. The Image J computer program was used for image analysis.

Relative amounts of LMP2 and LMP7 subunits in cleared thymus homogenates were detected by densities of corresponding bands on X-ray film. Graphs of density dependence on the amount of protein analyzed by Western blotting were plotted in advance. The range of protein amount in which the above-mentioned dependence was linear was chosen for further work.

Fixation and immunohistochemistry. Animals were perfused through the heart first with 0.1 M PBS until blood was washed off from blood vessels, then with 4% solution of paraformaldehyde in 0.1 M PBS for 15 min at 4°C. Then the thymus was isolated, fixed additionally in 4% paraformaldehyde at 4°C for 2 h, incubated in 15% sucrose solution in PBS for 24 h at 4°C, and frozen in isopentane at −40°C.

Twelve micron cryostat thymus sections were incubated successively in PBS with 0.1% Triton X-100 and 3% normal sheep serum for 30 min at 20°C, then with a mixture of primary antibodies (polyclonal antibodies to immune proteasome subunits LMP2 (1 : 2500) or LMP7 (1 : 2500) and monoclonal antibodies to T cell marker CD3 (1 : 500) or to cytokeratins CK10 (1 : 25), CK14 (1 : 10), CK18 (1 : 20), and CK19 (1 : 50), markers of thymus epithelial cells) for 18 h at 4°C, then with a mixture of secondary antibodies to rabbit and mouse IgG labeled, respectively, by CY3 (1 : 500) and FITC (1 : 100) in PBS for 2 h at 20°C. The specificity of antibodies was confirmed by controls in which the reaction was carried out in the absence of the primary antibodies. The absence of fluorescent label was indicative of reaction specificity.

The reaction was analyzed using a Leica DMRXA2 fluorescence microscope (Germany) equipped with an appropriate set of light filters and a digital camera.

Determination of apoptosis level in thymus. Apoptosis was evaluated in rat thymus at E18, E21, P7, P15, and P30 by staining fixed cells with propidium iodide and subsequent flow cytometric analysis. To obtain suspensions of thymus cells, rats were killed by cervical dislocation. Thymuses were homogenized in PBS at the temperature of thawing ice, filtered through a nylon mesh, and centrifuged at 400*g* for 10 min. The cells were fixed with cooled 70% ethanol and stored at −20°C for no more than one week before flow cytometry.

Fixed cells were washed with PBS and centrifuged for 10 min at 300*g*. The pellet was resuspended in 1 ml PBS containing RNase A (100 μg/ml) to destroy RNA and prevent propidium iodide binding to it. Cells were incubated at 37°C for 20 min, and then propidium iodide (1 μg/ml) was added. Staining was carried out at room temperature for 20 min in the dark. Intracellular DNA content was determined using a Coulter EPICS XL-MCL flow cytofluorometer. Fluorescence of at least 40,000 cells corresponding by light-scattering characteristics to viable ones was registered. The percentage of apoptotic cells (forming a hypodiploid region on a histogram) was determined on the basis of the data using the WinMDL program.

Statistical data processing was done using a nonparametrical Wilcoxon criterion.

RESULTS

Expression of the immune proteasome subunits LMP2 and LMP7 in rat thymus was estimated by Western blotting in fetuses at E18 and E21 and after birth at P1, P3, P5, P8, and P19 (Fig. 1). Immune subunits LMP2 (Fig. 1, a and c) and LMP7 (Fig. 1, a and b) were detected already at E18. At E21 and P1, their amount increased 2-4-fold and showed practically no change through P19.

Subunits LMP2 and LMP7 were also detected by immunocytochemistry in rat thymus of all age groups (Figs. 2 and 3). The largest amount was localized in the thymus stroma cells (Fig. 2, a and c). The use of double labeling by antibodies to the proteasome immune subunits and cytokeratins LMP2 and LMP7 subunits were found in epithelial thymus cells both in cortex and in medullar zone (Fig. 2, a and c).

The use of antibodies to the proteasome immune subunits and to the CD3 antigen of T lymphocytes made it possible to detect LMP2 and LMP7 subunits in thymus lymphocytes as well (Figs. 2b, 2d, 3a, and 3b). In this case, the amount of immunopositive material in lymphocytes was lower than that in epithelial cells.

In all studied thymus cell populations in rats of different age, immune proteasomes were localized in the cytoplasm. No visible distinctions were revealed between the LMP2 and LMP7 distribution in different thymus cell types.

The level of natural apoptosis in rat thymus was estimated in parallel at E18, E21, P7, P15, and P30 using flow cytometry.

In fetal thymus at E18 the level of apoptosis was 25%, then it decreased to 5% at E21 and remained at the same level until postnatal P30 (Fig. 4).

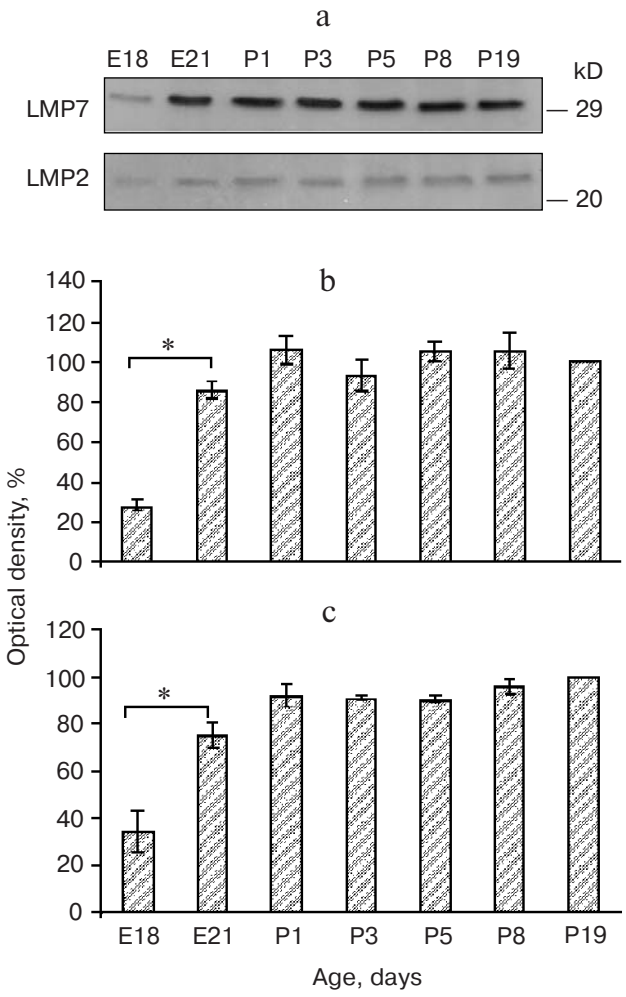

Fig. 1. Content of immune subunits LMP7 and LMP2 in cleared homogenates of rat thymus during prenatal (E18, E21) and early postnatal (P1, P3, P5, P8, P19) ontogeny. a) Western blots of proteins from cleared thymus homogenates using polyclonal antibodies to subunits LMP7 and LMP2. Markers: carboanhydrase (29 kD) and trypsin inhibitor (20 kD). b, c) Relative amounts (optical density of blots) of LMP7 and LMP2, respectively. The subunit content in thymus at P19 was taken as 100%. Mean values of 3-4 experiments are shown (± standard error of the mean), * $p < 0.05$.

DISCUSSION

In this work, possible molecular mechanisms involved in formation of the mammalian immunity T system during early ontogeny have been studied. We have shown that immune proteasomes play an important role in this process. Immune proteasomes contain the γ-interferon-inducible catalytic subunits LMP7 (β5i), LMP2 (β1i), and MECL1 (β2i) instead of catalytic subunits X (β5), Y (β1), and Z (β2) of constitutive proteasomes. The replacement of constitutive subunits by immune ones takes place during assembly of new proteasomes. In this case, subunits LMP2 and MECL1 are built in together but independently from LMP7. Although the LMP7 subunit incorporation into the newly formed proteasome is facilitated by the presence of LMP2 and MECL1, it is also possible without them [16, 17].

Unlike constitutive proteasomes regulating the content of intracellular proteins, the function of immune proteasomes is associated mainly with immunity. They participate in negative selection of thymocytes, in activation and differentiation of naive $CD8^+$ T lymphocytes to cytotoxic ones specific for certain antigenic epitopes, and in launching a signal concerning the deficiency of practically any cell [9, 18]. Immune proteasomes significantly increase the yield of potential antigenic oligopeptides with a "correct" C terminus containing residues of hydrophobic amino acids or arginine [19-21]. Antigenic oligopeptides of 8-11 amino acid residues are as a rule antigenic epitopes. Longer antigenic oligopeptides formed by immune proteasomes are shortened to the required length by aminopeptidases. This is due to the ability of oligopeptides, having such structure, to join in

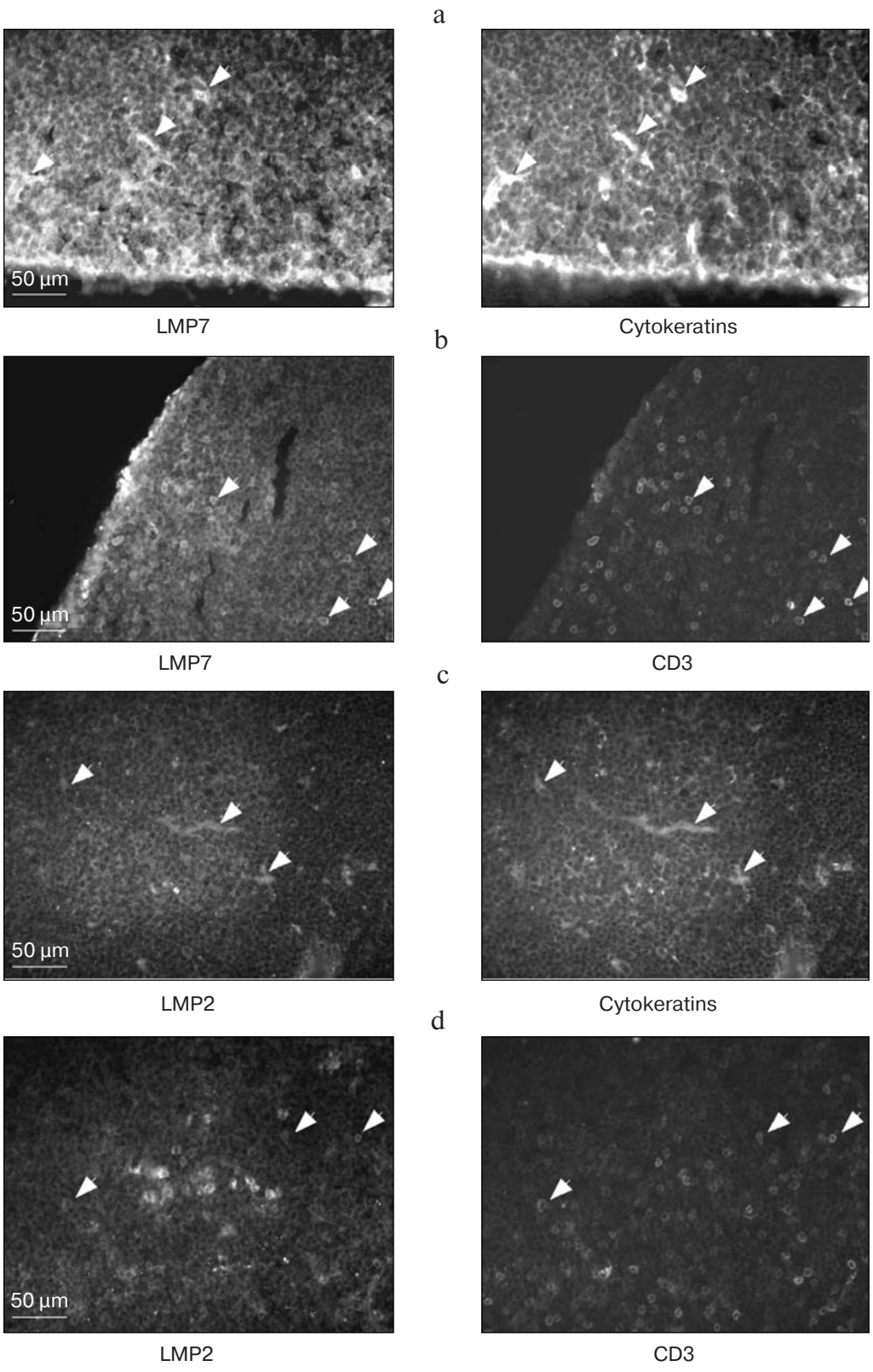

Fig. 2. Expression of immune subunits LMP7 and LMP2 in rat thymus cells at P30. a) Immunohistochemical detection of LMP7 and cytokeratins (markers of thymus epithelial cells); b) immunohistochemical detection of LMP7 and CD3 (marker of T lymphocytes); c) immunohistochemical detection of LMP2 and cytokeratins; d) immunohistochemical detection of LMP2 and CD3. Arrows point to the cells with double labels.

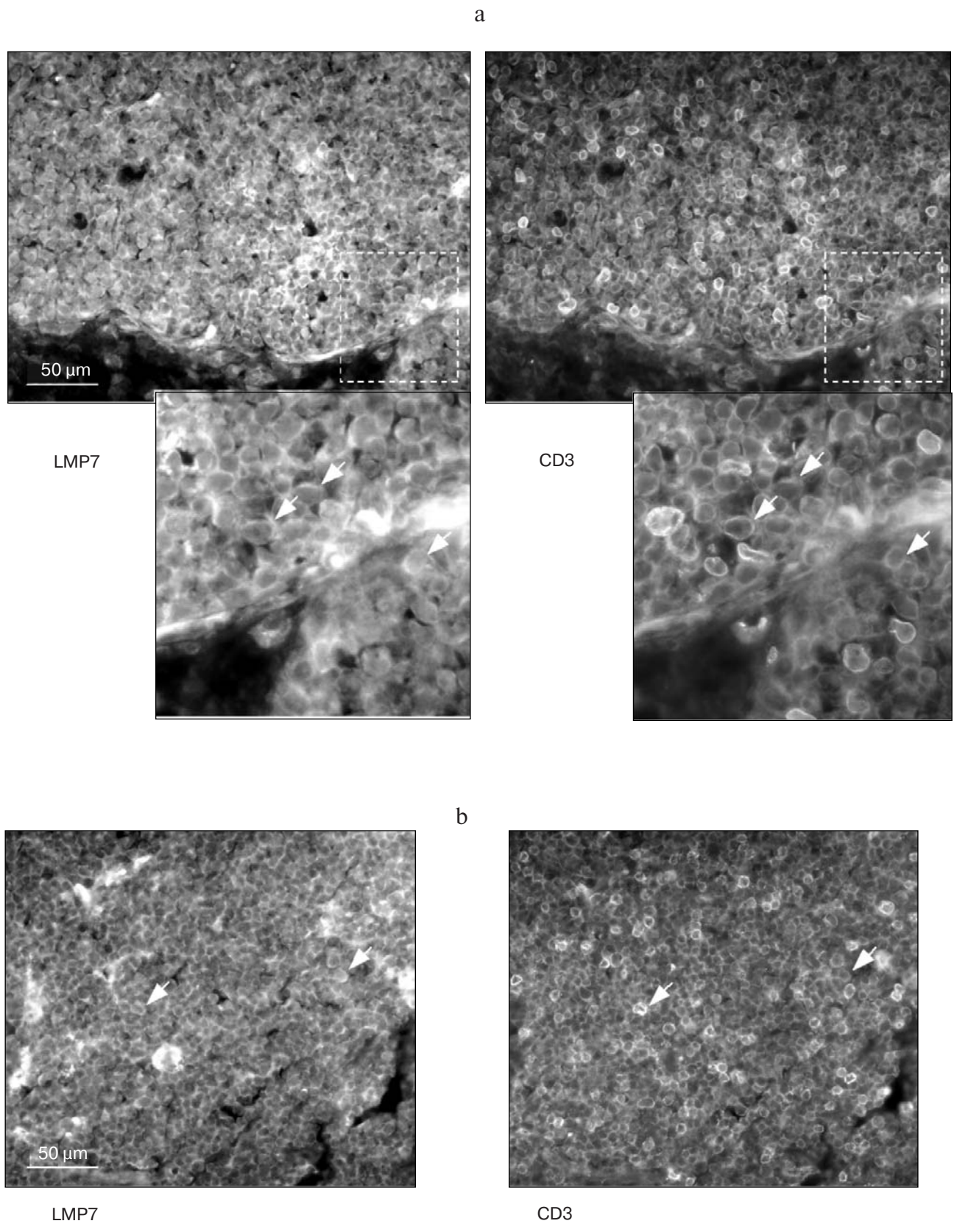

Fig. 3. Expression of immune subunits LMP7 in rat thymus cells at E21 (a) and P8 (b). Immunohistochemical detection of LMP7 and CD3 (marker of T lymphocytes). Arrows point to cells with double labels.

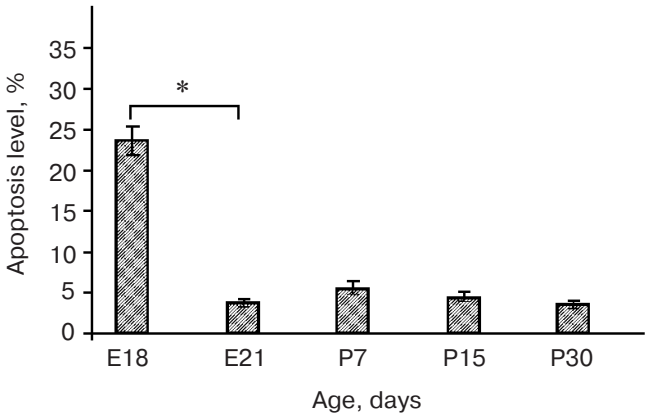

Fig. 4. Level of natural apoptosis in rat thymus cells during prenatal and early postnatal ontogeny. Days of embryonic (E18 and E21) and postnatal (P7, P15, and P30) development are shown on the abscissa axis. The ordinate axis shows the number of cells that entered apoptosis, expressed in % of total number of analyzed cells. Mean values ± standard error of the mean are shown (n = 6); * $p < 0.05$.

endoplasmic reticulum the class I MHC molecules by incorporation into Bjorkman's binding groove. The complex of antigenic oligopeptide with class I MHC molecules is exported onto the cell surface for recognition by the $CD8^+$ T lymphocytes [7, 22].

We have studied the dynamics of the content of LMP2 and LMP7 proteasome immune subunits in rat thymus during prenatal and early postnatal ontogeny. According to our data, immune proteasome subunits LMP2 and LMP7 are expressed in rat thymus already at E18. However, at this time their amount is low, and it increases 2-4-fold only by the time of delivery and then remains practically at the same level during following days. The LMP2 subunit expression level allows us also to judge about that of the LMP10 subunit co-incorporating into nucleosomes [16]. In thymus cells, as in studied cells of other organs, immune proteasomes are localized in the cytoplasm [23]. During embryonic and postnatal thymus development, immune proteasomes are localized mainly in epithelial (Fig. 2, a and c) and, probably, in dendritic cells, which will be the subject of our further investigations. The increase in the amount of immune proteasomes at the end of prenatal development is probably due to the fact that just at this stage there takes place active differentiation and formation of functionally mature epithelial cells of cortical and medullar thymus zones, which are able to provide for positive and negative selection [24, 25]. We have found immunopositive epithelial thymus cells both in cortical and medullar zones. However, no LMP2 and LMP7 subunits were found in mouse cortical epithelial cells [9]. This apparent contradiction might be caused both by interspecies differences and by different methodical approaches. The authors of the cited work revealed immune proteasomes in isolated cortical and medullar epithelial cells by Western blotting. In this case, the procedure of isolation of cell populations was long and included mechanical and enzymic dissociation followed by cytofluorometry. In our work, in addition to Western blotting, we have used an immunohistochemical method for detection of LMP2 and LMP7 on thymus sections, which seems to be more adequate because it shows more exactly the situation existing *in vivo*. According to data from the literature, in sexually mature species epithelial cells of the thymus cortical zone, which express class I or II MHC molecules, are involved in positive selection leading to accumulation of the T lymphocyte clone recognizing its own MHC molecules [26, 27]. Expression of immune proteasomes in cortical epithelial cells increases in infected animals or after injection of γ-interferon at physiological concentrations [9]. Medullar epithelial and dendritic cells participate in negative selection [9, 12, 28]. However, recent studies show that cortical epithelial cells and a small amount of cortical dendritic cells are involved in negative selection of CD8 and CD4 thymocytes [12, 28]. Thus, the question concerning localization in thymus of the process of negative selection is still open.

The combination of all these data suggests that immune proteasomes are involved in establishment of the process of negative selection in thymus already during prenatal ontogeny.

As we showed earlier [29], unlike thymus, the expression of immune proteasomes in the rat peripheral lymphoid organ spleen and in the non-lymphoid organ liver take place during postnatal development, where they have different functions.

Immune proteasomes are detected in spleen by Western blotting by the end of the first week after delivery, when all cellular components of the immune system capable of the immune response are present in this organ. In liver, immune proteasomes appear at later stages, by the 18-19th days of postnatal development. In the case of defective cell emergence, in liver cytotoxic T lymphocytes acquire the ability to migrate from the spleen with the blood and lymph and to eliminate these cells just in this period.

It should be noted that the expression level of immune proteasomes in thymus revealed by us during prenatal and early postnatal ontogeny appreciably exceeds that in the spleen during the first postnatal week [29]. These data also confirm an especially important role of immune proteasomes in the processes of negative thymocyte selection in thymus during embryonic development.

Analysis of age dynamics of the thymus natural apoptosis level also favors this hypothesis because it is known that thymocytes that did not pass through selection in thymus undergo programmed cell death [14, 15]. Like the level of immune proteasomes, the intensiveness of thymocyte apoptosis by the time of delivery reaches a stable level characteristic of postnatal animals (Fig. 4). It should be noted that a rather high level of apoptosis is reg-

istered in thymus at E18. Evidently, this is not associated with thymocyte selection, because the expression level of immune proteasomes at E18 is still very low [25]. Probably the high level of apoptosis at E18 is the result of active thymus occupation at that time by precursors of T lymphocytes [30, 31], while the amount of functionally mature recesses able to maintain their further differentiation is limited [24, 25, 32].

Immune proteasomes should generate autoantigens and foreign antigenic oligopeptides for following presentation to T lymphocytes; this function is not unique and, evidently, not the primary one.

We have also found a small amount (compared to epithelial cells) of immune proteasomes in rat thymocytes (Figs. 2b, 2d, 3a, and 3b). Expression of immune subunits, independent of induction by γ-interferon, was detected in various lymphoid cells, including T lymphocytes, in sexually mature rats and mice [9, 33, 34]. There are also data showing that in T lymphocytes these subunits are involved in regulation of cell proliferation. It was shown that T cells from mice with knocked out genes of subunits LMP7 and LMP10 exhibit hyperproliferative activity to polyclonal mitogens which is not associated with another function, namely, with generation of antigenic epitopes forming complex with class I MHC molecules [35]. This suggests that along with presentation of antigenic epitopes, immune proteasomes in thymocytes can also fulfill other important functions [18] like participation in production of biologically active peptides [35].

Thus, there are now a sufficient number of facts pointing to the involvement of immune proteasomes in immunity, but they require further analysis and generalization.

According to our data, expression of immune proteasomes, involved in the autoantigen presentations to thymocytes during negative selection in thymus, is observed both in the age of sexual maturity and in early ontogeny, and this process in rats is established during prenatal development.

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