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Peculiarities of Proteasome Pool Formation in Rat Spleen and Liver during Postnatal Development

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Abstract—Changes in the specific activity and amounts of 26S and 20S proteasome pools in rat spleen and liver during postnatal development and appearance in them of immune subunits were studied. Two decreases in chymotrypsin-like activity of the proteasome pools were recorded during the first three weeks after birth. The activity minimums fell on the 11th and 19th days, and the first decrease was more prolonged and pronounced than the second. The decrease in the specific activity of the 26S proteasome pools was associated with a reduction of their quantity. The 20S proteasome pools displayed no such decreases. Noticeable quantities of immune subunits LMP7 and LMP2 were revealed by Western blotting in the spleen on the 7th day and on the 19th day in the liver, concurrently with the beginning of the decrease in the proteasome activity. It was concluded that during the first three weeks of postnatal development the proteasome pools in rat spleen and liver were replaced twice, and in the spleen (a lymphoid organ) a qualitatively new pool containing immune subunits appeared nearly two weeks earlier than in the liver (a non-lymphoid organ). The appearance of immune proteasomes in different organs and tissues during some weeks after birth seems to explain the immune system inefficiency during embryogenesis and early postnatal development.

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Key words: immune and constitutive proteasomes, postnatal development

All organs and tissues of mammals contain multiple forms of proteasomes which are different in structure, type of protein hydrolysis, and, hence, physiological role. The 26S and 20S proteasomes are studied most comprehensively. The 26S proteasomes regulate cellular processes, such as replication and repair of DNA, transcription, signal transmission, cell cycle, immune response, and apoptosis via ATP- and ubiquitin-dependent degradation of proteins involved in these processes [1]. The 26S proteasomes are multisubunit protein complexes consisting of a proteolytic "core" (20S proteasomes) and two regulatory 19S subparticles and possessing a number of catalytic sites. By the sets of proteolytically active subunits, 26S proteasomes of mammals can be subdivided into constitutive and immune ones. The immune proteasomes contain 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 and perform certain functions during the immune response [2]. The LMP2 and MECL1 subunits are incorporated into newly produced proteasomes jointly but independently of LMP7. The LMP7 subunit incorporation is promoted by the presence of LMP2 and MECL1, but can be also realized in their absence [3, 4]. In this manner multiple subtypes of immune proteasomes are produced: the subtype containing all γ-interferon-inducible catalytic subunits, the subtype containing the LMP2 and MECL1 subunits, and the subtype containing only LMP7 which have been detected in some organs of mammals [5-7].

The 20S proteasomes are present in the cell not only as a part of 26S proteasomes, but also as independent particles and can hydrolyze some proteins damaged by oxidation independently of ubiquitin and ATP [8-10].

As proteasomes are involved in many cellular processes, it is reasonably to expect that functions of their pools are changing during different stages of ontogenesis of animals, and these changes have to be different in dif-

Abbreviations: PMSF) phenylmethylsulfonyl fluoride; TNT) Tris-HCl, NaCl, Tween-20.

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ferent organs and tissues. In fact, proteasomes have been shown to change during embryogenesis of drosophila, clawed frog, and chicken. These changes occur in the location [11, 12], quantity [13], and activity [14] of proteasomes. The changes in proteasome activity during chicken embryogenesis are different in the pectoral muscles, liver, and brain [14].

Proteasomes have been studied in the course of rat aging [15-17], and their activity was found to be changed in the liver [15, 16] and skeletal muscles [17] of old animals as compared to that in young ones. However, in work [15] proteasome activity in liver was found to increase, and in work [17] it was decreased [16]. In skeletal muscles, proteasome activity was increased [17]. It was suggested that the decrease in proteasome activity in liver should predetermine an increase in the half-life of proteins and its increase in skeletal muscles should reflect a decrease in muscular mass on aging. The proteasome activity in rat liver and skeletal muscles is regulated differently: changes in their activity in skeletal muscles correlated with changes in their quantity [17], whereas the latter remained constant in liver [15, 16], hence, changes in their activity should be associated with post-translational mechanisms.

The literature on changes in the proteasome pool during ontogenesis of animals is scarce and scattered. There are no data on proteasome functioning during postnatal development of mammals, which is coupled with intensive physiological and biochemical rearrangements in the organism. Therefore, we studied the proteasome pools in rat spleen (a lymphoid organ) and liver (a non-lymphoid organ) during postnatal development. We found earlier that the immune subunit LMP2 appeared in the spleen between the fifth and ninth days. In the liver, this subunit appeared later, between the 15th and 23rd days [18].

The present work was designed to study the course of appearance of the immune subunits LMP7 and LMP2 in these organs, and the proteasomes were studied in more detail (with interval of two days) during the first three weeks after birth. In addition, changes in the activity and quantity of 26S and 20S proteasomes during this period were studied.

MATERIALS AND METHODS

The liver and spleen of 5-23-day-old Wistar rats were used in the experiments.

Monoclonal antibodies to the Rpt6 subunit and subunits α1,2,3,5,6,7 and polyclonal antibodies to the immune subunits β1i (LMP2) and β5i (LMP7) of proteasomes were obtained from Affinity (Great Britain). An ECL kit, Hybond-ECL nitrocellulose membranes, and peroxidase-conjugated antibodies to mouse and rabbit IgG were from Amersham Biosciences (Great Britain). Suc-LLVY-AMC (N-succinyl-leu-leu-val-tyr7-amido-4-methyl coumarin) and MG132 (Z-leucyl-leucyl-leucinal) were from Sigma (USA).

Buffers. Buffer A: 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 5 mM $MgCl_2$, 2 mM ATP, 10 mM $Na_2S_2O_5$, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml).

Buffer B: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 20% glycerol, 5 mM $MgCl_2$, 2 mM ATP, 10 mM $Na_2S_2O_5$, 0.2 mM PMSF, leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml).

Buffer C: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 20% glycerol, 10 mM $Na_2S_2O_5$, 0.2 mM PMSF, leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml).

Buffer TNT: 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20.

Fractionation of proteasomes. All procedures were performed at 0-4°C. Liver and spleen pieces (0.6 g) were washed in a standard phosphate saline buffer and homogenized in a Braun Melsungen (Germany) homogenizer (glass–glass) in 5 ml of buffer A. The homogenates were centrifuged using a K-23 centrifuge (Janetsky, Germany) at 2000*g* for 15 min and then in an L7-55 ultracentrifuge (Beckman, USA) with an SW 50.1 rotor at 105,000*g* for 1 h. Proteins of the cleared homogenates were fractionated in two stages with $(NH_4)_2SO_4$. The fractions enriched with 26S and 20S proteasomes were prepared by addition of $(NH_4)_2SO_4$ to 40 and 70% saturation, respectively [19]. To the 40% saturation, $(NH_4)_2SO_4$ was added by portions for 20 min on a magnetic stirrer. On the complete dissolving of $(NH_4)_2SO_4$, the preparation was stirred for another 20 min and then centrifuged at 12,500 rpm using a Heraeus (Germany) table-top centrifuge. The precipitate was dissolved in 250 μl of buffer B. For convenience, we shall denote this fraction as the 0-40% fraction. The supernatant was supplemented with $(NH_4)_2SO_4$ to 70% saturation. The salting-out was performed as described above. The precipitate was dissolved in 250 μl of buffer C (the 40-70% fraction).

Determination of proteasome activity. Proteasome activity was determined by hydrolysis of the fluorogenic oligopeptide Suc-LLVY-AMC [20]. The reaction mixture for determination of the 20S proteasome activity contained 20 mM Tris-HCl (pH 7.5), 1 mM DTT, and 30 μM Suc-LLVY-AMC. To determine the activity of 26S proteasomes, the reaction mixture was additionally supplemented with 5 mM $MgCl_2$ and 1 mM ATP. The reaction was performed at 37°C for 20 min after the introduction of 0.5-2 μl of the 0-40% or 40-70% fraction into the reaction mixture (to the final volume of 100 μl) and stopped by addition of 1% SDS. The resulting product was recorded with a fluorimeter at the excitation wavelength of 380 nm and the emission wavelength of 440 nm. The

quantity of proteasomes providing for hydrolysis of 1 nmol Suc-LLVY-AMC during 1 min was taken as the activity unit. The specific activity was determined in units per mg protein of the cleared homogenate.

Western blotting. After SDS-PAGE (0.3 mg protein per lane) in 13% gel, the polypeptides were semi-dry transferred from the gel onto a nitrocellulose membrane. The membrane was incubated for 2 h at 20°C in TNT buffer, then for 1 h in TNT buffer containing 5% defatted milk and monoclonal antibodies to the Rpt6 subunit or subunits α1,2,3,5,6,7 (or polyclonal antibodies to the immune subunit LMP2 or LMP7) diluted 1 : 2500, washed several times in the TNT buffer and incubated for 1 h in the TNT buffer containing 5% defatted milk, and peroxidase-conjugated antibodies to mouse (or rabbit) IgG diluted 1 : 10,000. After the washing in TNT buffer, the membrane was treated routinely using the ECL system.

Other methods. Polyacrylamide gel electrophoresis of proteins in the presence of SDS was performed by the Laemmli method [21]. The protein concentrations in the fractions were determined by the Lowry method [22]. The density of bands on X-ray film was evaluated using the Image J standard computerized program.

RESULTS

Separation of the 26S and 20S proteasome pools. The activity and quantity of proteasomes in different organs and tissues of animals are usually studied using crude extracts [14, 23]. This seems to be due to destruction of 26S proteasomes into 20S and 19S subparticles during purification, but this distorts the results. Such works can give an idea about the total activity and quantity of proteasomes in the objects under study. To follow changes in the 26S and 20S pools of proteasomes during rat postnatal development, we had to create a method for their separation providing for the maximum retention of the intact structure of 26S proteasomes. We have shown earlier that 26S and 20S proteasomes of rat liver can be isolated at 40% (the 0-40% fraction) and 70% saturation (the 40-70% fraction), respectively, of the cleared homogenate with $(NH_4)_2SO_4$. By subsequent gel filtration on Sepharose-2B, the 0-40% fraction was found to contain an admixture (~30%) of 20S proteasomes, whereas the 40-70% fraction was free from admixture of the 26S proteasomes [19] and, consequently, could be used for studies on the 20S proteasome pool. But there was a question, whether the admixture of 20S proteasomes in the 0-40% fraction was a contamination with the native 20S proteasomes or a product of the 26S proteasome destruction. To answer this question, we changed either conditions of the 26S proteasome fractionation with $(NH_4)_2SO_4$ (to the cleared homogenate $(NH_4)_2SO_4$ was added to 38 and 42% of saturation) or those of the gel filtration of the 0-40% fraction (the gel filtration time was several-fold increased). The 20S proteasome admixture in the 0-38 and 0-42% fractions (~30%) was approximately the same as in the 0-40% fraction, but it markedly increased (to 70 and 90%) in the 0-40% fraction if the filtration time was increased 1.5- and 2-fold, respectively, and this was accompanied by a sharp decrease in the activity of the 26S proteasomes (unpublished data). These findings suggested that the admixture of 20S proteasomes in the fraction 0-40% should more likely be a result of destruction of the 26S proteasomes than the contamination with native 20S proteasomes. The 26S proteasomes seemed to be destroyed as a result of interaction with Sepharose-2B during gel filtration. It might be expected that the fraction 0-40% not subjected to gel filtration should contain <30% or no admixture of 20S form, which would make it suitable for studies on the pool of 26S proteasomes.

Appearance of immune subunits LMP7 and LMP2 in pools of 26S and 20S proteasomes from rat spleen and liver during postnatal development. The contents of immune subunits LMP7 (30 kD) and LMP2 (23 kD) in the proteasome fractions from spleen and liver were studied by Western blotting using polyclonal antibodies to these subunits (Fig. 1). By the content of the LMP2 subunit, the content of the jointly incorporated MECL1 subunit was also evaluated. By this technique, noticeable amounts of the two immune subunits, LMP7 and LMP2, were detected in spleen on the 7th day after birth (Fig. 1, blots *1-4*) in 26S proteasome fraction (Fig. 1, blots *1* and *3*) and also in the fraction of 20S proteasomes (Fig. 1, blots *2* and *4*). In liver small amounts of these two subunits appeared only on the 17th day of postnatal development (Fig. 1, blots *5-8*), and on the 19th day their contents increased, and as in the spleen they were constituents of both 26S (Fig. 1, blots *5* and *7*) and 20S proteasomes (Fig. 1, blots *6* and *8*).

The next purpose of this study was to find out how the specific activity and content of the 26S and 20S proteasome pools were changing on appearance in them of qualitatively new, immune, subunits.

Changes in specific activities of 26S and 20S proteasome pools in rat spleen and liver during postnatal development. The activities of 26S and 20S proteasomes were determined by hydrolysis of the fluorogenic oligopeptide Suc-LLVY-AMC utilized by the chymotrypsin-like sites in the fraction 0-40 and 40-70%, respectively. To exclude the contribution of the admixed proteolytic activity, the inhibitor MG132 of chymotrypsin-like sites of proteasomes was used. The final calculations were performed as the difference between the total and residual activities in the presence of 5 μM MG132. Figure 2 presents the changes in the specific activities of the 26S and 20S proteasome pools in rat spleen and liver from the 5th to 23rd days of postnatal development. The specific activity is expressed in units per mg protein of the spleen and liver cleared homogenates. The results (Fig. 2) suggest a common tendency for an increase in the activities of both 26S

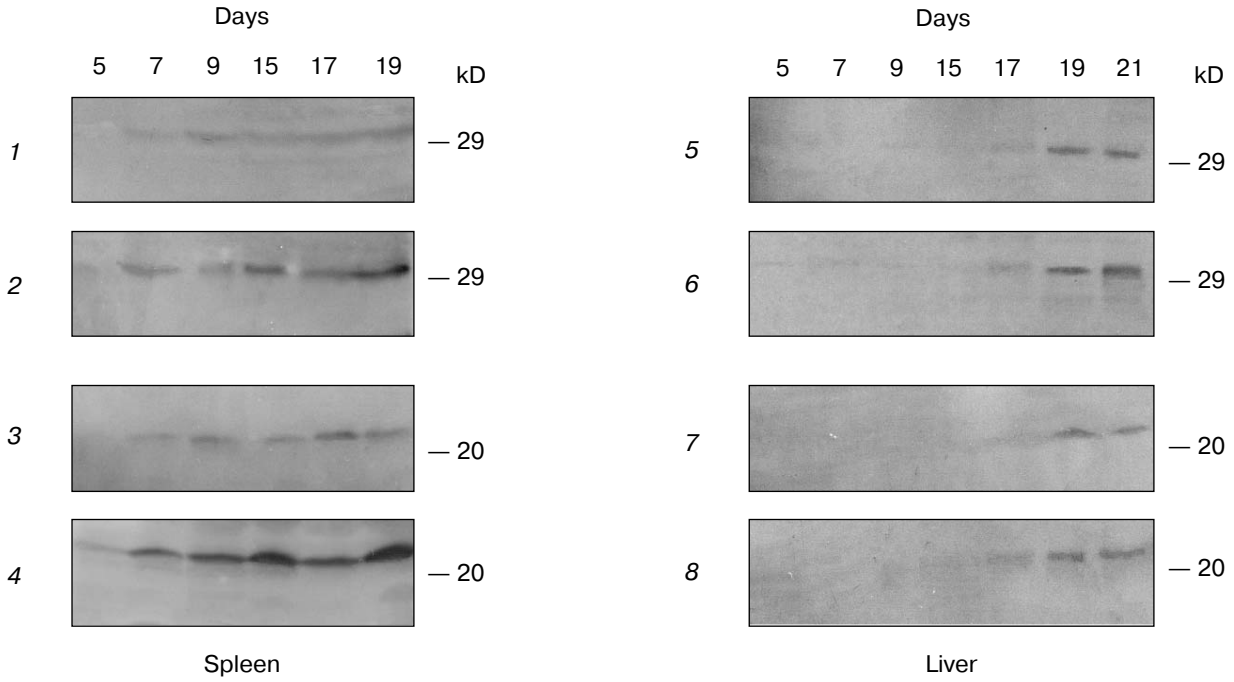

Fig. 1. Western blots of proteins from fractions of the 26S proteasomes (*1*, *3*, *5*, *7*) and 20S proteasomes (*2*, *4*, *6*, *8*) isolated from spleen (*1-4*) and liver (*5-8*) of different age rats. Polyclonal antibodies to the subunits LMP7 (*1*, *2*, *5*, *6*) and LMP2 (*3*, *4*, *7*, *8*) were used. Carboanhydrase (29 kD) and trypsin inhibitor (20 kD) were used as markers.

and 20S proteasomes in the two organs studied by the 23rd day as compared to the activities on the 5th day. On this background, two periods of decreased activity were detected in both pools of proteasomes in the spleen and liver. The first period began from the 7th day and continued longer than the second period. The minimum activities were recorded in the 11- and 19-day-old rats. Note that noticeable amounts of the immune subunits LMP7 and LMP2 in either organ appeared at the same time as the activities of the 26S and 20S pools of proteasomes began to decrease (on the 7th day in the spleen and 19th day in the liver).

Changes in quantity of 26S and 20S proteasome pools in rat spleen and liver during postnatal development. The relative quantity of the 26S proteasome pools in the spleen and liver was evaluated on different stages of development by Western blotting of proteins from the 0-40% fraction using monoclonal antibodies to the Rpt6 subunit (46 kD) which is a constituent of the regulatory subunit 19S (Fig. 3a, blots *1* and *3*). The relative quantity of the 20S proteasome pools was determined in the 40-70% fraction by the same method using monoclonal antibodies to the subunits α1,2,3,5,6,7 (29-32 kD) which produce α-rings (Fig. 4a). After the detection of specific bands on the X-ray film by the standard chemiluminescence reaction, their densities were determined and calculated per mg protein of the cleared homogenates. The results are presented in Figs. 3b and 4b. The changes in the quantity of the 26S proteasomes in rat spleen and liver

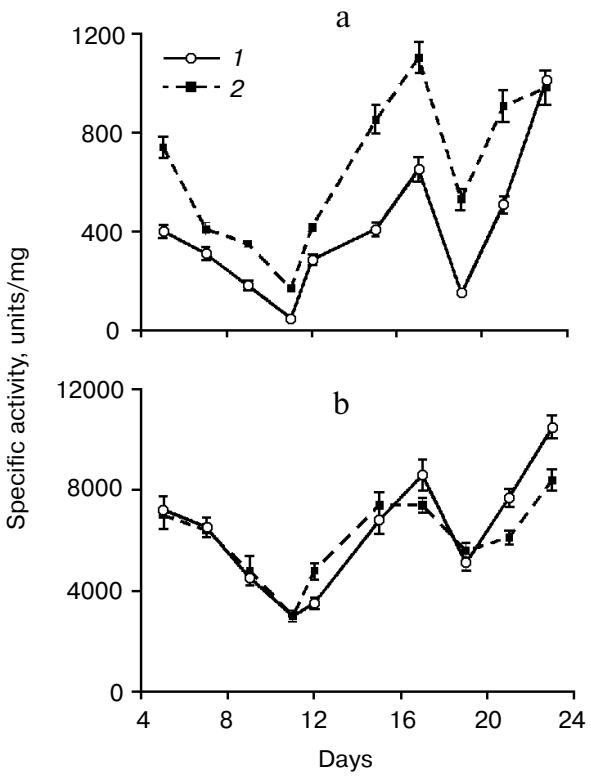

Fig. 2. Specific activities of pools of 26S proteasomes (a) and 20S proteasomes (b) isolated from spleen (*1*) and liver (*2*) of different age rats. Here and in Figs. 3 and 4 for all points of the curves, the confidence interval is given at the significance level $p < 0.05$.

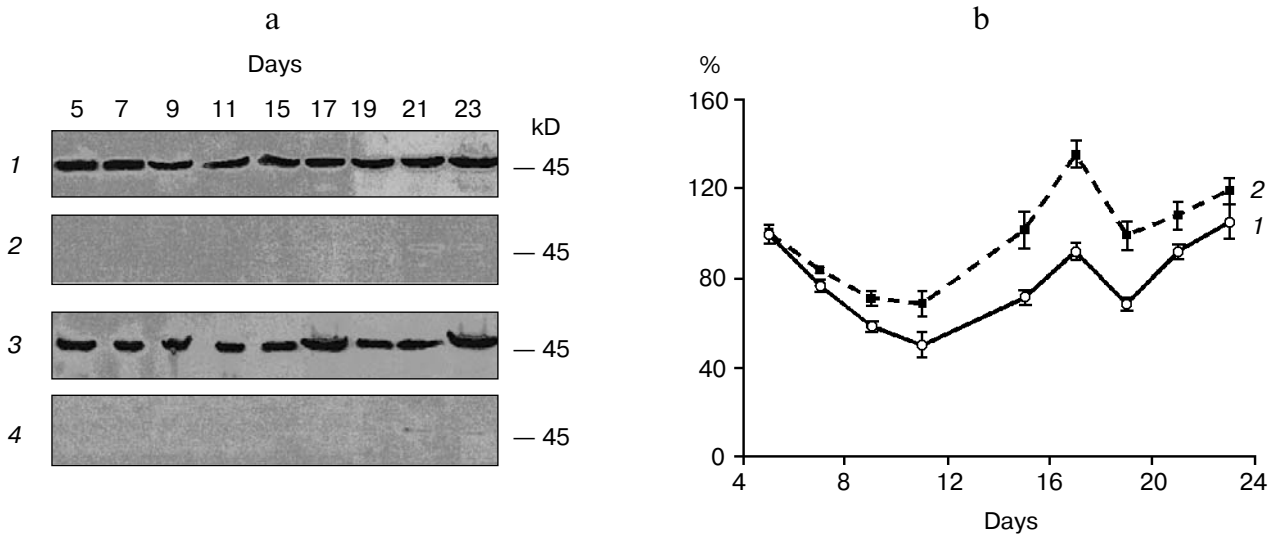

Fig. 3. Content of Rpt6 subunit in fractions of spleen and liver of different age rats. a) Western blots of proteins from the fractions of 26S proteasomes (*1*, *3*) and 20S proteasomes (*2*, *4*) isolated from spleen (*1*, *2*) and liver (*3*, *4*) using monoclonal antibodies to the Rpt6 subunit. Ovalbumin (45 kD) was used as a marker. b) The relative amount of the Rpt6 subunit in the fractions of 26S proteasomes isolated from spleen (*1*) and liver (*2*). The quantity of Rpt6 in the organs of 5-day-old rats was taken as 100%.

during postnatal development were similar to the changes in their specific activity: the decreases in the activity and contents of proteasomes were coincident, although there was no strict correlation between these values (Figs. 3b and 2a). Another pattern was observed for the pools of 20S proteasomes: their amounts in both spleen and liver did not significantly vary during different stages of development (Fig. 4b).

It should be noted that the Rpt6 subunit was virtually not detected in the fractions of 20S proteasomes (Fig. 3a, blots *2* and *4*), which indicated the absence of contamination of the 20S proteasome pools with the 26S form. The small quantity of the Rpt6 subunit detected in the fractions of 20S proteasomes on the 21st and 23rd days was likely to belong to 19S subunits not associated with proteasomes.

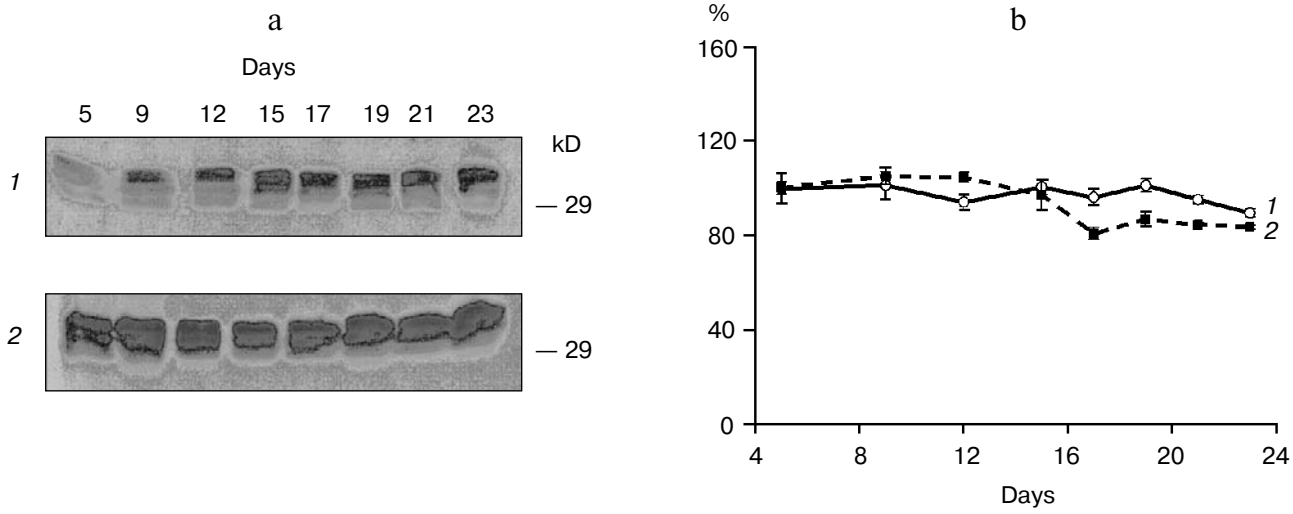

Fig. 4. Contents of α1,2,3,5,6,7 subunits in spleen and liver fractions of different age rats. a) Western blots of proteins from the fractions of 20S proteasomes isolated from spleen (*1*) and liver (*2*) using monoclonal antibodies to the α1,2,3,5,6,7 subunits. Carboanhydrase (29 kD) was used as a marker. b) The relative amount of the α1,2,3,5,6,7 subunits in the fractions of 20S proteasomes isolated from spleen (*1*) and liver (*2*). The quantity of α1,2,3,5,6,7 subunits in the organs of 5-day-old rats was taken as 100%.

DISCUSSION

The present work has revealed changes occurring during postnatal development in the pools of 26S and 20S proteasomes from rat spleen and liver. These changes are both similar and different (table). On one hand, the specific activity of all proteasome pools studied manifested two decreases during the first three weeks after birth (Fig. 2). The minimum activities in these periods were observed on the 11th and 19th days, and the first period of the decrease was more prolonged and characterized by lower activity. On the other hand, the decrease in the specific activity of the 26S proteasome pools was associated with a decrease in their amounts (Figs. 2a and 3), whereas no such pattern was observed for the pools of 20S proteasomes (Figs. 2b and 4).

Obviously, the parallel decreases in the activity and amount of 26S proteasomes is a result of exhaustion of the stored pools. The 26S proteasome pools stored in the embryonal spleen and liver begin to decrease on the 7th postnatal day, concurrently with the appearance of the immune subunits in the fraction of 26S proteasomes from the spleen (Fig. 1, blots *1* and *3*). In other words, on the 7th day, the reverse process begins of generation of new pools of 26S proteasomes, and in spleen this pool has a new quality because it contains immune subunits, whereas in the liver a new pool of constitutive 26S subunits is produced. Twelve days later (on the 19th postnatal day) the 26S proteasome pools are changed again, along with decreases in their activity and quantity and also the appearance of immune subunits in the liver (Fig. 1, blots *5* and *7*). Note, that the half-life of proteasomes is 12-15 days [24], which is consistent with our findings. The absence of a strict correlation between changes in the specific activity and contents of the 26S proteasome pools can be explained by the simultaneous realization of several processes, such as removal of the old pools and formation of the new ones, changes in their subunit composition, and the effects of regulatory proteins. Thus, the replacement of constitutive catalytic subunits by immune ones is known to be associated with changes in the proteasome activity [7, 25].

It should be noted that our finding of the consecutive periods of the decrease and increase in the proteasome activity during rat ontogenesis allows us to explain the contradictory data obtained in two laboratories for changes in the proteasome activity in rat liver during aging [15, 16]. It is very likely that in one case the activity was determined during the period of its decrease and in the other case it was measured during the period of its increase.

The replacement of the 20S proteasome pools in rat spleen and liver during the postnatal development seems to occur concurrently with the replacement of the 26S proteasome pools, which is shown by similar changes in the proteasome activity (Fig. 2) and appearance in them of immune subunits (Fig. 1). The lack of a detectable change in the amount of 20S proteasomes (Fig. 4) does not contradict this assumption and is explained by specific features of their assemblage: the formation of 20S proteasomes begins from formation of their inactive precursors [26]. In general, different changes in the contents of the 26S and 20S proteasome pools reflect the difference in the mechanisms of their formation: 20S proteasomes are produced consecutively from separate subunits, whereas 26S proteasomes are produced from the ready 20S and 19S subparticles. Note that the 20S proteasomes of rat spleen and liver are different from the 20S proteasomes of mouse organs by the presence of immune subunits [7]. The immune subunits have been revealed in the fractions of 20S proteasomes also in the liver of adult rats [27]. It seems that the immune 20S proteasomes form stores in the rat organs, which in the case of necessity can change to functional 26S proteasomes capable of being involved in the immune response.

The immune 26S proteasomes are responsible for at least two functions in the adaptive T-cellular immune

Changes in the pools of 26S and 20S proteasomes in rat spleen and liver during early postnatal development

Parameter	Patterns of changes in pool of proteasomes			
	26S		20S	
	in spleen	in liver	in spleen	in liver
Chymotrypsin-like activity	decreases twice, beginning from the 7th and 19th days	decreases twice, beginning from the 7th and 19th days	decreases twice, beginning from the 7th and 19th days	decreases twice, beginning from the 7th and 19th days
Amount	decreases twice, beginning from the 7th and 19th days	decreases twice, beginning from the 7th and 19th days	does not change	does not change
Immune subunits	appears by the 7th day	appears by the 17-19th day	appears by the 7th day	appears by the 17-19th day

response of mammals [2]. First, they produce or initiate the production of antigenic epitopes in the cytoplasm of the cells synthesizing viral or mutant endogenous proteins in all organs and tissues except the brain. The antigenic epitopes are transported into the endoplasmic reticulum where they form complexes with the class I molecules of the Major Histocompatibility Complex (MHC), and these complexes are transferred onto the cell surface. Cytotoxic T-lymphocytes recognize these structures and cause apoptosis of defective cells. Second, immune proteasomes in the antigen-presenting cells of the immune system in the lymphoid organs are also responsible for another function: they are involved in the activation of naive T-CD8$^+$ lymphocytes that results in their proliferation and differentiation to cytotoxic T-lymphocytes specific to definite antigenic epitopes. It has been shown in the present work that in the rat spleen immune proteasomes appear earlier (by the end of the first week after birth) than in the liver where they can be detected only on the third week (on the 17-19th day). By this time (on the 15-18th day), T-lymphocytes located earlier in the spleen migrate into the periarterial regions of the white pulp and form PALS-structures (Periarteriolar Lymphoid Sheath) or muffs (unpublished data). In other words, on appearance of defective cells in the liver which is under the immune defense of the spleen, cytotoxic T-lymphocytes can be transferred from the spleen white pulp by the blood and/or lymph flow into the liver and destroy these cells by the 17-19th day of postnatal development. The spleen seems to be ready for the T-cell immune response inside itself even on the earlier stages of development, already by the end of the first week after birth when immune proteasomes are produced in it and T-lymphocytes and other cells of the immune system are already present. The organism's ability for earlier provision of immune defense of the spleen as the immune system organ seems to be important for the overall immunity formation.

The present work opens new lines in studies on the immune system development during ontogenesis of mammals. Our data on the appearance of immune proteasomes in different organs within a few weeks after birth seem to explain the cause of immune system inefficiency during embryogenesis and early postnatal development. However, the findings generate some questions: are immune proteasomes produced in all cells of the spleen and liver? During what stages of ontogenesis are immune proteasomes produced in other lymphoid and non-lymphoid organs? And what is the ratio between the immune and constitutive proteasomes? We expect to answer these questions in our further studies.

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