Effects of Low-Molecular-Weight Thymic RNA on T Cell-Dependent Antibody Formation

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We proposed a method for isolation of low-molecular-weight RNA from mammalian thymus. Physicochemical properties and regulatory activity of these RNA in immunological test of T cell-dependent antibody formation were evaluated.

Key Words: *thymus; low-molecular-weight RNA; bioregulation; partial nucleolysis; T cell-dependent antibody formation*

Mechanisms of bioregulation are one of the most interesting biological problems. Protein substances as regulatory factors were the objective of many studies [10]. At the same time, there are data on the involvement of RNA in bioregulation, in particular, in immunoregulation [1,6,7]. Our previous *in vivo* and *in vitro* experiments showed that exogenous RNA modulates transcription. The most pronounced effect was produced by low-molecular-weight RNA (lmwRNA, ~8.5-3S) formed after fragmentation (autolysis) of high-molecular-weight cytoplasmic RNA (cRNA) [2]. We hypothesized that *in vivo* regulatory effects of RNA are mediated by mechanisms similar to those described by G. I. Chipens *et al*. for protein molecules [10]. The three-level theory of bioregulation postulates that proteins are synthesized in the form of inactive precursors and then are transformed into bioactive molecules after partial proteolysis. Partial proteolysis of these molecules results in their fragmentation with the formation of low-molecular-weight regulatory molecules. Biosynthesis of RNA in cells is accompanied by similar changes. It can be hypothesized that products of partial proteolysis, which are formed *in vitro* after RNA autolysis [3] and *in vivo* during apoptosis [15], play an important role in intra- and intercellular regulation. lmwRNA from the thymus, the major organ of the immune system, probably act as immunoregulators.

Here we isolated and characterized lmwRNA from the thymus and evaluated their ability to mediate immune reactions during T cell-dependent antibody formation.

MATERIALS AND METHODS

RNA from bovine thymus was isolated by the modified phenol and detergent methods. The thymus was minced, pumped through spinnerets, and mixed with buffer solution (0.15 M sodium citrate, pH 6.5-7.0) on ice. The homogenate was centrifuged at 1140*g* and 4°C for 15 min and the supernatant was collected. In series I (phenol method), an equivalent volume of phenol and 0.5% sodium dodecyl sulfate was added. The mixture was shaken for 15 min and centrifuged at 4500*g* and 4°C for 15 min. Proteins were removed by treatment with phenol, sodium dodecyl sulfate, and chloroform. RNA was precipitated with ethanol (3-fold volume).

In series II (detergent method) cold supernatant was added under constant stirring to the extracting solution (0.15 M NaCl, 0.015 M sodium citrate, 13% ethanol, and 1% sodium dodecyl sulfate) at 90°C. Temperature was brought to 75°C and then sharply decreased to 4°C. The mixture was centrifuged at 4500*g* and 4°C for 30 min. The precipitate was removed. Ethanol (2-fold volume) and 0.2 M NaCl were added

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to the supernatant. The mixture was kept at 4°C for 16 h to obtain the precipitate. Then the mixture was centrifuged at 4500*g* and 4°C for 15 min. The supernatant was removed. The precipitate was washed with 67% ethanol and 0.2 M NaCl and centrifuged at 4500*g* and 4°C for 15 min. The precipitate was dissolved in a buffer containing 0.15 M NaCl and 0.015 M sodium citrate (pH 6.5-7.0) and centrifuged at 43,700*g* and 4°C for 60 min. The precipitate was removed. Ethanol (3-fold volume) and 0.2 M NaCl were added to the supernatant. To obtain the precipitate this mixture was kept at 4°C for 1 night and then centrifuged at 4500*g* and 4°C for 25 min. The supernatant was removed. The precipitate was dissolved in a minimum amount of distilled water. Ethanol (3-fold volume) and 0.2 M NaCl were added. These manipulations were repeated twice.

RNA from rat thymus and mouse liver and RNA autolysates were obtained as described previously [3]. The composition of preparations was determined [2].

The size of ribonucleic fragments was evaluated by electrophoresis in 2.5 and 8% polyacrylamide gel (PAAG) under denaturing conditions [6]. We used 0.1 M Tris-borate electrode buffer. The gels contained 7 M urea. The samples were denatured by heating in formamide containing 7 M urea at 65°C for 3 min. Electrophoresis was performed at 100 V and 55 mA (2.5% PAAG) or 400 V and 15 mA (8% PAAG) for 1.5 h.

Immunological activity of RNA preparations was estimated by local hemolysis described by N. K. Gerne and A. A. Nordin [12]. The mice were intraperitoneally immunized with 3% suspension of sheep erythrocytes (0.05 ml) and received subcutaneously 0.1 ml isotonic NaCl (control) or test preparation (experiment). The preparation was used in doses of 1, 10, and 100 μg. The mice were killed 5 days after treatment.

The results were analyzed by Mann—Whitney test.

RESULTS

Previous studies showed that phenol extraction on ice allows to isolate the total fraction of cRNA containing high-molecular-weight 28S and 18S ribosomal RNA, lmwRNA 5.8S, 5S, and 4S RNA, and minor components from cells of Ehrlich's ascites carcinoma and mouse liver. The procedure of autolysis proposed by

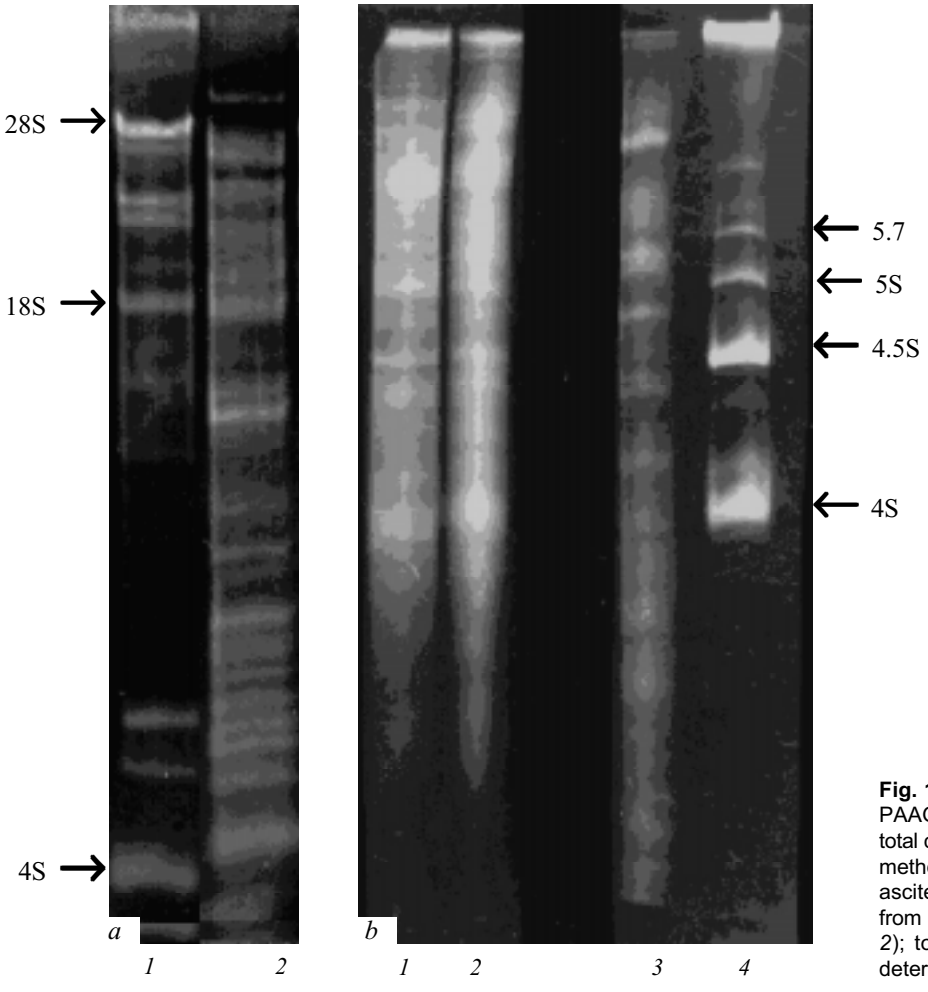

Fig. 1. Electrophoresis of RNA in 2.5 (*a*) and 8% PAAG (*b*). *a*) Total cRNA from mouse liver (*1*); total cRNA from mouse thymus obtained by phenol method (*2*). *b*) Autolysate of cRNA from Ehrlich's ascites carcinoma (120 h, *1*); autolysate of cRNA from rat thymus obtained by phenol method (72 h, *2*); total RNA from bovine thymus obtained by detergent method (*3*); marker lmwRNA (*4*).

TABLE 1. Contents of RNA, DNA, and Protein in Preparations Obtained by Phenol and Detergent Methods (%)

Method	RNA	DNA	Protein
Phenol	90.0	8.5	1.5
Detergent	90.0	5.0	5.0

us results in fragmentation of high-molecular-weight RNA. Autolysates obtained by this method contain the mixture of lmwRNA (8.5S-2S RNA).

However, after treatment of the thymic tissue by this method the content of cell nuclei (including DNA) passed into the aqueous phase, which makes further purification of RNA impossible. The method with modifications allowed us to obtain cRNA with a satisfactory degree of purity from freshly isolated rat and mouse thymus tissues (Table 1). Electrophoresis showed that the content of 28S RNA in preparations from the thymus is lower than in liver cRNA (Fig. 1, *a*, zone *1*) Preparations from the thymus contained some additional components, which were also detected in trace concentrations in liver cRNA (Fig. 1, *a*, zone *2*). Thus, isolation of RNA from the thymus tissue is accompanied by partial autolysis of high-molecular-weight RNA. Autolysis of cRNA from Ehrlich's ascites carcinoma and liver cells yields autolysates with identical and reproducible spectra of components (Fig. 1, *b*, zones *1*, *2*). These data suggest that *in vitro* autolysis is accompanied by specific partial nucleolysis. Highly polymeric rRNA molecules are cleaved by structurally determined sites. Our previous studies [2-4] and published data indicate [5,15] that these changes are similar those observed *in vivo*. rRNA fragments probably possess regulatory properties. lmwRNA from the thymus can mediate the immune response.

Previous studies revealed immunological activity in preparations of lmwRNA from the thymus. The peptide preparation activin T and new immunotropic ribonucleic compound Ribotim (RT) were obtained from the thymus. We developed a detergent method for isolating RT from frozen bovine thymus tissues. This procedure is much more efficient than the phenol method. The amount of RNA obtained by the detergent method is sufficient for studying biological activity. Preparations obtained by the phenol and detergent methods contained similar amounts of RNA, but differed in the composition of admixtures (Table 1).

RT contained lmwRNA with molecular weights of 56-13 kDa (as estimated by calibration curves, Fig. 1, *b*). We compared the composition of RT and autolysates of native preparations from the thymus and Ehrlich's ascites carcinoma (Fig. 1, *b*, zones *1-3*). The preparation obtained by the detergent method contained not only lmwRNA found in autolysates, but also components with a lower molecular weight (<4S RNA). Therefore, the detergent method allowed us to obtain lmwRNA from the thymus without additional autolysis. It should be emphasized that the spectra of components are high reproducible, if RNA isolation from the thymus is performed with scrupulous attention to the proposed method. The preparation isolated by the detergent method was used to estimate biological activity of RT.

Treatment with 1 and 10 μg RT dose-dependently increased the count of antibody-producing cells (APC, $p<0.01$, Fig. 2, *a*). Increasing the dose of RT above 10 μg practically did not potentiate this effect. RT pre-

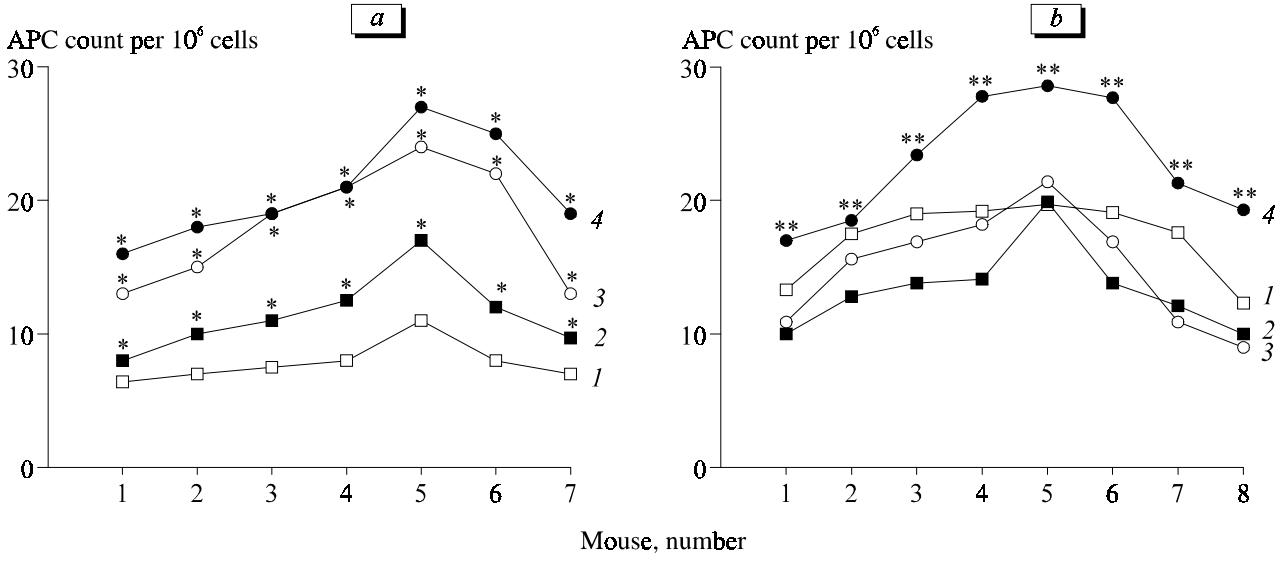

Fig. 2. Immunological activity (Gerne test, count of antibody-producing cells, APC) of Ribotim containing 56-12 kDa components (*a*) and components below 14 kDa (*b*). Control (*1*) and Ribotim in doses of 1 (*2*), 10 (*3*), and 100 μg (*4*). *$p<0.01$ and **$p<0.05$ compared to the control.

parations of various series caused similar changes (Table 2). The index of stimulation was highly reproducible during treatment with 1 μg RT, but varied after administration of RT in doses of 10 and 100 μg.

This is probably related to the fact that RT preparations of various series were tested in different mice. Our results indicate that the effects of RT were dose-dependent: despite considerable variations, RT in doses of 1 and 10 μg produced different effects.

In some experimental series RT was fractionated on columns packed with Sephadex G-100 and Sepharose 6B (data not shown). The fraction containing components with molecular weights below 14 kDa possessed no immunological activity (1 and 10 μg) or produced only minor stimulatory effects (100 μg, stimulation index 1.2, Fig. 2, *b*,). The effects of fractions with a higher molecular weight were similar to those of the preparation (Fig. 2, *a*). Thus, there is a lower limit of the size of lmwRNA stimulating the specific immune response.

Therefore, partial nucleolysis of high-molecular-weight cRNA from the thymus leads to the formation of functionally active fragments (RT) *in vivo* modulating T cell-dependent antibody formation. Our results and published data that RT modulates delayed-type hypersensitivity, wound healing in mice, leukocyte mobility, and reparation after experimental burn traumas in rats [7-9] indicate that preparations of lmwRNA from the thymus exhibit immunobiological activity. The mechanisms of these effects and tissue specificity of RT require further investigations.

TABLE 2. Immunological Activity of RT Preparations Obtained in Various Series (Gerne Test, Stimulation Index)

Experiment	RT dose, μg/mouse		
	1	10	100
1	1.4	2.3	2.7
2	1.4	3.0	3.0
3	1.3	3.1	2.4
4	1.6	1.8	2.0
5	1.5	1.7	2.2
6	1.3	1.6	1.8
$X \pm s$	1.4±0.1	2.2±0.3	2.3±0.2

Note. Significant differences between RT in doses of 1 and 10 μg ($p<0.05$).

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