Study of Protein Components of Natural Peptide Regulators

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Special attention should be given to the safety of drugs prepared from animal products. Industrial production of cytomedines and cytamines includes treatment of organs and tissues from young animals. Studies of preparations by methods recommended by the World Health Organization indicate that this technology guarantees the absence of infectious agents, protooncogenes, nucleic acids, and prion proteins.

Key Words: *prion proteins; protooncogenes; histochemistry; immunoblotting*

Animal products can include infectious agents, protooncogenes, and nucleic acids. Prion infections caused by prions (specific infectious proteins belonging to amyloids) attract much recent attention [1,2,6,9]. During industrial production of preparations isolated from animal organs and tissues, special attention is given to removal of admixtures (*e.g.*, infectious agents) from the active substance.

Natural preparations of two classes, cytomedines (medicinal drugs) and cytamines (parapharmaceutics), were synthesized at the St. Petersburg Institute of Bioregulation and Gerontology [3,5].

Here we studied the composition of cytomedines and cytamines to confirm the absence of their contamination with functionally active protooncogenes, viable viruses, and prion proteins.

MATERIALS AND METHODS

The technology for production of cytomedines and cytamines provides maximum protection from contamination with infectious agents. The active substance is isolated from animal organs and tissues at 3-7°C and pH 3.0 for 6 days. These conditions are sufficient for complete inactivation of viruses even without additional treatment [8].

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Expert verification of contamination of peptide regulators with nucleic acids was performed by their phenol-detergent extraction from the preparation and electrophoresis in 1% agarose gel in Tris-acetate buffer (staining with ethidium bromide) [4].

Samples of preparations (200-fold concentrated) were analyzed by histochemical methods, electron microscopy assay, electrophoresis, and immunoblotting to confirm the absence of prion proteins in peptide regulators.

The test preparations were analyzed by polyacrylamide gel (PAAG) electrophoresis to evaluate the content and molecular weight of proteins.

Primary screening for prion infections was performed histochemically. Test preparations were stained with Congo red. This method allows detecting amyloid proteins and prions in the preparation.

Cytomedines and cytamines are water-soluble preparations. Therefore, samples were fixed in PAAG (not on a glass). Histological preparations of human amyloid liver served as the positive control.

Immunoblotting was performed using polyclonal antibodies to mammalian PrP proteins.

For electron microscopy, concentrated fractions of cytomedines and cytamines were placed on a formvar/carbon films (sorption time 10 min) and examined under a JEM-100 S electron microscope (JEOL, ×15,000-100,000). The films with sorbed preparations were subjected to proteolytic treatment with proteinase K (0.2 mg/ml at 37°C for 5, 10, and 20 min), contrasted, and examined under a microscope for the pre-

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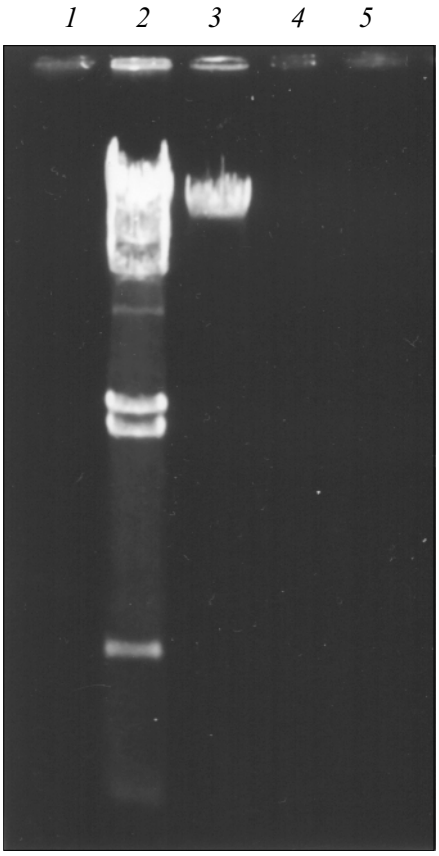

Fig. 1. Electrophoresis of cytomedines in 1% agarose gel after phenol-detergent extraction: negative control (0.9% NaCl, *1*), molecular weight marker (lambda phage DNA treated with restriction endonuclease Hind III, *2*), native DNA from human blood lymphocytes (positive control), Cortexin (*4*), and Retinalamin (*5*).

sence of proteolytically resistant scrapie associated fibrils (SAF), including prions.

RESULTS

Nucleic acids were not detected in Cortexin and Retinalamin (Fig. 1). These results could be expected taking into account the method for obtaining the preparations. Ultrafiltration on hollow fibers with a resolution of 15 kDa guarantees retention of most nucleic acids. These particles contain more than 45 (single-stranded molecules) or 23 nucleotides (double-stranded molecules). Fragments of DNA and RNA containing no more than 45 nucleotides do not possess matrix or infectious activity [7,8].

These data indicate that cytomedines and cytamines do not include viable viruses or functionally active protooncogenes.

Staining for total protein showed that Cortexin and Retinalamin contain only short polypeptides and polyamines with a molecular weight of no more than 10 kDa. This value is much lower than the molecular weight of prions (Fig. 2). Thus, electrophoresis confirmed the absence of high-molecular-weight proteins (*e.g.*, prions) in cytomedines.

Staining of concentrated cytomedines and cytamines fractions fixed in PAAG with Congo red revealed no selective binding of the dye. However, the preparation from human amyloid liver was characterized by strong selective staining with Congo red and yellow-green luminescence of amyloid fibrils.

Therefore, histochemical assay revealed no amyloid-like protein compounds (*e.g.*, prions) in peptide bioregulators.

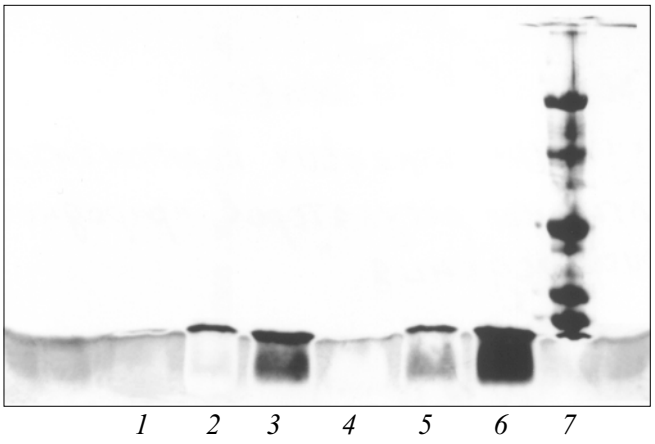

Fig. 2. Electrophoresis of cytomedines in PAAG: Retinalamin in concentrations of 1 (*1*), 5 (*2*), and 10 mg/ml (*3*), Cortexin in concentrations of 1 (*4*), 5 (*5*), and 10 mg/ml (*6*), and standard solution of marker proteins (*7*).

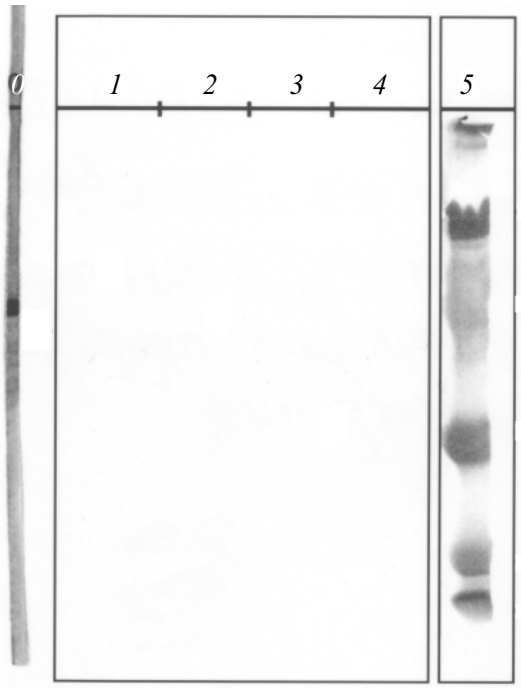

Fig. 3. Immunoblotting analysis of cytomedines with antibodies to PrP proteins: Retinalamin in concentrations of 5 (*1*) and 10 mg/ml (*2*), Cortexin in concentrations of 5 (*3*) and 10 mg/ml (*4*), and standard marker proteins (*5*).

Immunoblotting revealed no binding of antibodies to preparations from animal organs and tissues (Fig. 3). Thus, normal cellular PrP proteins present in raw materials were eliminated during industrial production of preparations. The final product did not contain these proteins. Abnormal PrP proteins (prions) were not detected.

Direct electron microscopy of prions (SAF) in combination with proteolysis allows detection of these proteins by proteolytic resistance and morphological characteristics of fibrillar structures formed by prions.

Some authors observed aggregation of individual prion molecules with the formation of thread-like structures (SAF) after treatment with proteinase K in low concentrations. In some instances prions cannot be detected by electron microscopy before treatment with proteinase.

In our experiments fibrillar structures similar to prions were not revealed (even after proteolysis).

Diagnostic techniques recommended by the World Health Organization, immunoblotting study, and electron microscopy revealed no amyloid compounds (*e.g.*, prions) in cytomedines and cytamines.

Our results indicate that the technology for production of natural bioregulators cytomedines and cytamines guarantees the absence of functionally active protooncogenes, viable viruses, and other infectious agents, including prion proteins.

REFERENCES

1. V. A. Zuev, I. A. Zavalishin, and V. M. Roikhel', *Prion Diseases of Humans and Animals* [in Russian], Moscow (1999).
2. O. I. Kiselev, P. A. Nekrasov, O. Yu. Reshetnikova, and A. A. Vorob'ev, *Epidemiol. Infects. Bolezni*, No. 1, 4-9 (1998).
3. B. I. Kuznik, V. G. Morozov, and V. Kh. Khavinson, *Cytomedines: Experience of 25-Year Experimental and Clinical Studies* [in Russian], St. Petersburg (1998).
4. A. V. Mazin, K. D. Kuznedelov, and A. S. Kraev, *Methods of Molecular Genetics and Genetic Engineering* [in Russian], Novosibirsk (1990).
5. V. G. Morozov, G. A. Ryzhak, and V. V. Malinin, *Cytamines (Bioregulators of Cellular Metabolism)* [in Russian], St. Petersburg (1999).
6. *Problems of Health Care Related to Spongiform Encephalopathies in Humans and Animals. Memorandum to the Conference of the World Health Organization* [in Russian], Byull. VOZ, **70**, No. 2, 24-31 (1992).
7. F. Fenner, B. Mak-Oslen, and S. Mims, *Biology of Animal Viruses* [in Russian], Moscow (1977), Vol. 1, pp. 75-77.
8. F. Fenner, B. Mak-Oslen, and S. Mims, *Biology of Animal Viruses* [in Russian], Moscow (1977), Vol. 2, p. 293.
9. S. B. Prusiner, *Semin. Virol*., **7**, 157-167 (1996).