densities of the media were measured at 415 nm, and the rates of formation of the oxidation product – formaldehyde – in the presence and in the absence of the inhibitor were calculated.

In the in vivo experiments, 0.1% solutions of the substrates in 20-50% dimethyl sulfoxide were injected intraperitoneally into mice in doses of 10 mg/kg 30 min before the administration of hexobarbital soluble (40 mg/kg subcutaneously). The prolongation of the sleep of the mice as compared with the control amounted to 20 ± 3.6 min.

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IMMUNOAFFINITY FRACTIONATION OF NEUTRALIZING POLYCLONAL ANTIBODIES TO NERVE GROWTH FACTORS

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A method of fractionation according to affinity and specificity of polyclonal antibodies to murine and ovine nerve growth factors (NGFs) has been developed. The antibody preparations obtained neutralize the biological activity of β-NGF at a molar ratio of about 1 and recognize the species-specific and conservative epitopes of these NGFs, being valuable probes for two-site immunoanalyses.

Nerve growth factors (NGFs) are polypeptide growth factors with a broad spectrum of biological action which regulate the establishment, development, and functioning of individual populations of neurons of the peripheral and central nervous systems [1-4]. A considerable part of the investigations of NGFs is based on the use of antibodies, obtained most frequently against NGFs isolated from classical sources (mouse submaxillary salivary glands [5]. the venoms of certain snakes [6-8], and bovine seminal plasma [9]).

In spite of the wide use of monoclonal technology, polyclonal antisera are continuing to be widely used, their advantages being the effective neutralization of biological activity and also a usually higher affinity of the specific antibodies. In the present paper we describe a method for fractionating clonal antibodies with respect to affinity and specificity which permits high-affinity fractions of antibodies to species-specific and conservative epitopes of NGFs to be obtained.

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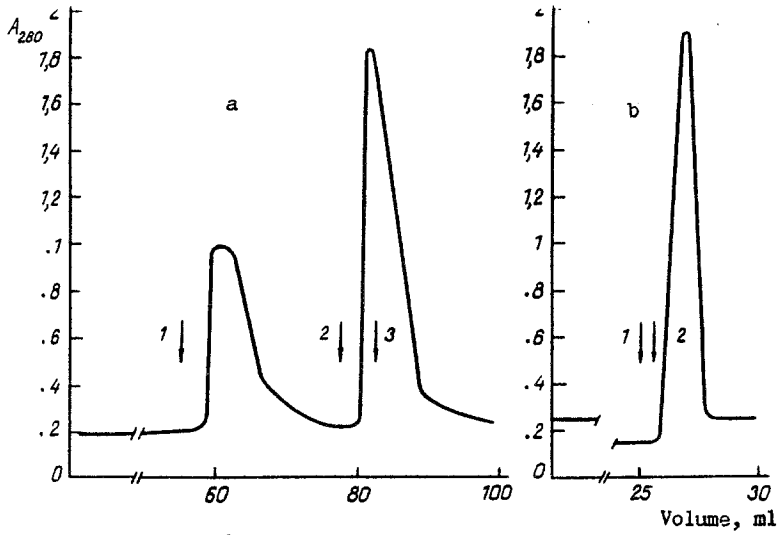

Fig. 1. Graphs of the immunopurification of antibodies. a) Purification of the antibodies on β-NGF bound to agarose. The stages of depositing the fractionated antiserum and washing the column with the starting buffer (TBS) are now shown: 1) 0.2 M CH_3COONa, pH 4.5; 2) 20 mM HCl in 0.15 M NaCl, pH 1.9; 3) the starting buffer. b) Purification of antibodies on bovine NGF bound to agarose. The steps of depositing the high-affinity fraction of antibodies to β-NGF and the subsequent washings with the starting TBS containing 0.2% of Tween-20 and distilled water are not shown: 1) 20 mM HCl in 0.15 M NaCl, pH 1.9; 2) starting buffer.

Fractionation was carried out by the method of double immunoaffinity purification of an antiserum to the β-subunit of murine NGF on the immobilized β-NGF and on immobilized bovine NGF.

A scheme of the purification of antibodies to murine β-NGF (β-ABs) is shown in Fig. 1. Intermediate elution with 0.2 M CH_3COONa at pH 4.5 completely eliminated ballast proteins and the low-affinity fraction of the antibodies. The high-affinity fraction was obtained by elution at pH 1.9. The purity of the latter fraction was determined by analytical rechromatography on a column of fresh β-NGF-agarose with the same elution scheme. The absence of unbound protein on the washing of the column with the starting buffer and also on elution at pH 4.5 confirmed the immunochemical purity of the last chromatographic peak (not shown). It can be seen from the ratios of the areas of the peaks that the predominating part of the specific antibodies of the antiserum was represented by the high-affinity fraction. The quality of the antibodies obtained was evaluated from the suppression of the activity of the NGF in a standard biotest [10] on the sensory ganglia of seven- to nine-day chick embryos in the presence of 1 biological unit (BU) of β-NGF (5-10 ng/ml) or of bovine NGF (2-4 ng/ml). Curves of the titration of the β-ABs are given in Fig. 2.

The β-ABs completely suppressed the maximum growth of the neurites caused by 1 BU of β-NGF (5-10 ng/ml), in a concentration of 25-50 ng/ml, i.e., at a molar ratio of NGF to antibodies of about 1.

The neutralization of 1 BU of bovine NGF (2-4 ng/ml) was achieved at a β-AT concentration of 3.2-6.4 μg/ml.

The β-ABs were subjected to further purification on a column of immobilized bovine NGF. The doubly purified preparation of antibodies so obtained (2-ABs) were tested analogously. The titration curve of the neutralizing activity with respect to β-NGF remained practically identical with the former one, while in relation to the bovine NGF an eightfold purification was achieved (neutralizing concentration 0.4-0.8 μg/ml) (Fig. 2).

The preparation of the highly purified antibodies to NGFs was connected with certain difficulties. In view of the laboriousness of the procedure for purifying the factor and the high cost of its commercial preparations, the immobilization of the NGFs was carried out at a fairly low protein/matrix ratio, which unavoidably led to an increase in nonspe-

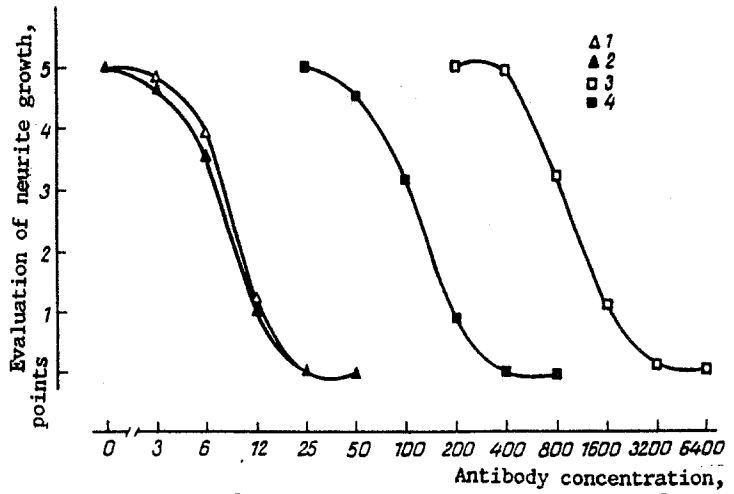

Fig. 2. Titration of the neutralizing activity of antibodies to β-NGF and the antibodies from double purification in the biotest on the spinal ganglia of 7- to 9-day chick embryos in the presence of 1 BU of β-NGF (5 ng/ml) or of bovine NGF (2 ng/ml): 1) β-NGF, antibodies to β-NGF; 2) β-NGF, antibodies from double purification; 3) bovine NGF, antibodies to β-NGF; 4) bovine NGF, antibodies from double purification. The biological effect was evaluated on the standard five-point scale (0 – absence of neurite growth; 5 – maximum neurite growth). Each point represents the mean value from two independent experiments.

cific sorption. NGF itself also sorbs a number of serum proteins nonspecifically [11]. In the initial experiments we carried out the purification of the antibodies directly from hyperimmune serum and did not include intermediate washings of the immunosorbent. In the electrophoretic analysis of the preparations obtained, impurities, mainly albumin, were usually detected. In light of what has been said, in the final scheme of purification we used only the $(NH_4)_2SO_4$ fractionation of the antiserum, and also performed additional washing with an intermediate medium at pH 4.5 and a fairly high ionic strength. This is an important improvement in comparison with the scheme of purification frequently used [12].

The procedure for immunoaffinity purification that has been developed has permitted us for the first time to obtain a preparation of polyclonal antibodies to NGF with the maximum possible neutralizing activity according to the results of highly sensitive (2.3 pM) biological testing.

The yield of doubly purified antibodies was not more than 12% of the amount of antibodies from single purification deposited on the immunosorbent with the bovine NGF, which corresponds to an 8-fold decrease in the neutralizing titers of the doubly purified antibodies in relation to the bovine NGF. Such a low immunological affinity of the two NGFs indicates that they have substantial conformational differences, in spite of the high homology of the nucleotide sequences of mammalian NGFs [13] and agrees well with the report of Cattaneo et al. on the presence of a biospecific immunodominant epitope in the region of the active center of the β-NGF molecule [14]. Thus, the predominating part of the antibodies from single purification is directed against the biospecific epitopes of the β-NGF, including determinants present in the immediate vicinity of the active center.

The doubly purified antibodies were specific for the common determinants of murine and bovine NGFs and, presumably, this preparation contained antibodies against the evolutionarily conserved active center of the NGFs. In actual fact, on biotesting, this antibody preparation inhibited the neurite-forming effect of the venoms of several Central Asian snakes (Vipera lebetina, Echina multisquamatus, and Naja oxiana) and of the monitor (Varanus griseus) saliva, and also an enriched NGF fraction from the blood serum of healthy donors (these results will be published separately). It may be assumed that further fractionation on immunosorbents with snake NGFs will permit to a considerable extent the sorting out of antibodies directed to the active centers of the molecules.

Thus, we have obtained high-affinity antibodies to the species-specific and common epitopes of murine and bovine NGFs possessing the maximum possible neutralizing activity

with respect to β-NGF and being valuable probes for monitoring the levels of the factor in tissues and biological fluids. A combination of doubly purified antibodies with singly purified antibodies not bound by bovine NGFs will permit an additional increase in the specificity of β-NGF in two-site systems of immunoanalysis.

EXPERIMENTAL

We used highly purified NGFs from murine salivary glands (7S and the β-subunit) from the Institute of Physiology of the Belorussian SSR Academy of Sciences, Minsk; bovine seminal plasma NGF from the Institute of Applied Molecular Biology of the USSR Ministry of Health, Moscow; Freund's adjuvant from Calbiochem and the Pacific Ocean Institute of Bioorganic Chemistry, Far Easter Division of the USSR Academy of Sciences; bovine serum albumin (BSA), fraction V, from Sigma; BrCN-activated agarose from Kemotex, Tallinn; and domestic reagents of analytic purity. Protein in eluates was recorded by a Uvicord II UV detector (LKB). The samples were concentrated in a Minicon B15 cell (Amicon).

Production of Antisera to Murine NGF. A monitor lizard was immunized subcutaneously with 1 mg of the high-molecular-mass 7S NGF in 0.15 M NaCl emulsified in a double volume of complete Freund's adjuvant. Intensifying injections were carried out with 1 mg of NGF in incomplete Freund's adjuvant five times with 5-week intervals. In the last injection, 0.5 mg of the subunit of the NGF was used. The titers were tested by Ouchterlony double diffusion in 1.5%-agarose plates. Blood was collected on the 7th day after the last injection and was kept at room temperature for 4 h, and then the serum was separated off by centrifugation and was lyophilized.

Preparation of the Immunosorbents. Following the instructions of the manufacturers of the activated agarose, 4 mg of the β-unit of murine NGF and 0.5 mg of bovine NGF were bound with BrCN-activated agarose in a ratio of 1 mg of protein per 1 ml of gel. The column was equilibrated with 20 mM Tris–140 mM NaCl, pH 7.5 (TBS) and then with 20 mM HCl–140 mM NaCl, pH 1.9, and again with TBS. The rate of elution was 12 ml/h for deposition and 35 ml/h for the washings. Chromatography was conducted at room temperature.

Purification of the Antibodies to the β-Subunit of the NGF. The antiserum was reconstituted in the initial volume of distilled water and was fractionated with $(NH_4)_2SO_4$ at 50% saturation; the precipitate was collected by centrifugation, and dialysis was carried out against two changes of TBS (500 volumes, 20 g each). The diffusate corresponding to 10 ml of the antiserum was deposited on a column of β-NGF-agarose equilibrated with the starting TBS and this was washed with 10 volumes of TBS and 5 volumes of 0.2 M CH_3COONa, pH 4.5, after which the antibodies were eluted with 5 m of 20 mM HCl–140 mM NaCl, pH 1.9. The eluate was neutralized immediately to pH 7 and was further dialyzed against TBS and stored at −10°C or was subjected to further purification on a second affinity column.

Purification of Antibodies to the NGF from Bovine Seminal Plasma. The antibodies obtained from the first column were passed through a column containing bovine NGF immobilized on agarose and equilibrated with TBS. The column was washed with 20 volumes of TBS containing 0.2% of Tween 20 and with 5 volumes of distilled water, and the antibodies were eluted with 20 mM HCl–140 mM NaCl, pH 1.9. The antibodies were neutralized and were concentrated in the Minicon B15 cell overnight at 4°C, followed by dialysis against TBS. The protein content of the diffusate was determined by the method of Lowry et al. [16] with BSA as standard. The antibodies were stored at −10°C before use.

Testing of the Biological Activities of the NGFs and Antibodies. Biotesting in a culture of the sensor ganglia of 7- to 9-day-old chick embryos was carried out in a culture medium containing 47% of medium 199 and 47% of Hanks' medium supplemented with 6% of bovine serum, 250 units/ml of penicillin, and 100 units/ml of streptomycin [10]. The results of the biotests were read after cultivation at 37°C for 18-21 h. Under these conditions the maximum growth of neurites (1 biological unit, BU) was observed at concentrations of 5-10 ng/ml of β-NGF and 2-4 mg/ml of bovine NGF. The sensitivity of the biotest is 60 pg/ml of β-NGF (2.3 pM). In the experiments on the suppression of NGF activity by antibodies, the NGF was kept with various concentrations of antibodies in the culture medium at room temperature for 4-5 h before addition to the ganglia.

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CHARACTERISTICS OF THE GLUCOSE ISOMERASE FROM Streptomyces atratus

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The molecular mass (Mr) of highly purified glucose isomerase from Streptomyces atratus has been determined. The native glucose isomerase, which has affinity for xylose, consists of an oligomeric protein containing 1552 amino acid residues and formed of four subunits. The enzyme is stable in the range of temperatures from 40 to 80°C and at pH values of from 6.0 to 11.0.

It is known DS-Na is capable of causing the dissociation of many proteins [1-5]. To achieve complete dissociation, the enzyme was treated with 1% DS-Na containing 0.1% of 2-mercaptoethanol. The dissociation products formed under these conditions were separated by electrophoresis in PAAG containing DS-Na, and also by the method of sedimentation equilibrium in an ultracentrifuge.

Since in the present of detergents the molecular mass of the enzyme decreases by a factor of approximately four, it was assumed that the St. atratus glucose isomerase consists of four subunits. The molecular mass of the St. atratus glucose isomerase with DS-Na, determined by the sedimentation equilibrium method was 43 kDa, and its sedimentation constant was 4.8S.

When the enzyme under investigation was treated with 6 M and 8 M urea, a marked decrease in enzymatic activity was observed. The molecular mass of this enzyme, determined by thin-layer electrophoresis in PAAG in the present of 6 M urea, was likewise 40 kDa.

A comparison of the amino acid composition of the St. atratus glucose isomerase with those of enzymes isolated from other sources showed similarity with respect to the number of residues of hydrophobic and acidic amino acids [7-9]. Table 1 shows the amino acid composition of the St. atratus enzyme. It can be seen that, in this enzyme, residues of acidic amino acids (glutamic and aspartic acids) and of hydrophobic amino acids (alanine and leucine) predominate over those of basic amino acids (arginine and lysine). The assumption exists that the predominance of hydrophobic over hydrophilic amino acids will lead to a

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