AUTOIMMUNE RESPONSE TO INJECTION OF A PROTEIN PREPARATION FROM BOVINE CAUDATE NUCLEI INTO RABBITS

V. A. Smirnov, V. I. Ivanskii, and A. N. Panyukov

UDC 612.017.1.014.46:615.361.813.2

The plasma membranes of bovine caudate nuclei were solubilized with the aid of Triton X-100. Membrane proteins extracted by the detergent were fractionated by chromatography on DEAE-cellulose and the affinity adsorbent suggested for separation of nicotinic cholinergic receptor protein (N-ChR) from the electric organ of Torpedo. Immunization of rabbits with the purified protein preparation caused half of them to develop a syndrome of myasthenia type. This effect resembles the autoimmune response of the electric organ of the fish to N-ChR. The presence of an N-ChR in the caudate nuclei possessing similar antigenic determinants to the N-ChR of electric organs and skeletal muscles is postulated.

KEY WORDS: caudate nuclei; affinity chromatography; nicotinic cholinergic receptor; myasthenia

It was shown a few years ago that immunization of rabbits with preparations of cholinergic receptor protein of nicotinic type (N-ChR) from the electric organ of fishes leads to the development of a syndrome of myasthenia type in them [8, 10]. Subsequent investigations showed that the cause of the disease is blocking of the N-ChR of the neuromuscular junction by antibodies formed to the N-ChR of the electric organ [5, 6]. The phenomenon thus discovered enables the hypothesis of the autoimmune mechanism of development of myasthenia gravis to be tested experimentally [2, 12]. So far an autoimmune response of myasthenia type has been obtained only in rabbits immunized with N-ChR from the electric organ of the electric eel and Torpedo. It was therefore interesting to study the possibility of isolating an N-ChR inducing a similar phenomenon from other sources and, in particular, from the brain. The content of cholinergic protein in the tissues is known to correlate with the concentration of acetylcholinesterase (AChE). Our attention was accordingly drawn to the caudate nuclei of the mammalian brain, which possess very high AChE activity. However, only a cholinergic receptor protein of muscarinic type could be detected in these structures by pharmacological methods [1].

This paper gives data on the isolation of a protein preparation from the caudate nuclei of the bovine brain which, when injected into rabbits, induces an allergic reaction of myasthenia type similar to that produced by N-ChR from the electric organ of fishes. The preparation was isolated by means of an adsorbent specific for N-ChR and suggested for purification of the N-ChR from the electric organ of the electric eel [9].

EXPERIMENTAL METHOD

Bovine brains were obtained from an abattoir. All the operations to isolate the caudate nuclei, fractionate the tissue, and isolate the protein preparations were undertaken at 4-6°C. A 10% homogenate in 0.32 M sucrose was prepared from the caudate nuclei by means of a Potter's homogenizer with Teflon pestle (clearance about 0.1 mm). The homogenate was centrifuged at 1000g for 10 min. The resulting supernatant was centrifuged at 25,000g for a further 30 min. The residue thus formed was treated with 9 parts of distilled water, homogenized, and centrifuged at 105,000g for 45 min. The loosely bound proteins were removed by homogenization of the membranes in 2 M NaCl followed by centrifugation. The membranes were then washed with distilled water and homogenized in a 3% solution of the nonpolar detergent Triton X-100 in 0.01 M Na-phosphate buffer, pH 7.4. One volume of residue of the membranes was treated with 2 volumes of the detergent solution. The membranes were extracted on a magnetic mixer for 1 h and then centrifuged at 105,000g for 1 h. The supernatant was diluted 5 times with phosphate buffer and applied to a DEAE-cellulose column (1.6 × 7 cm). The bound protein was eluted with 0.5 M NaCl in phosphate buffer and, after dilution, was applied to a column

Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR S. N. Golikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 84, No. 12, pp. 687-690, December, 1977. Original article submitted April 5, 1977.

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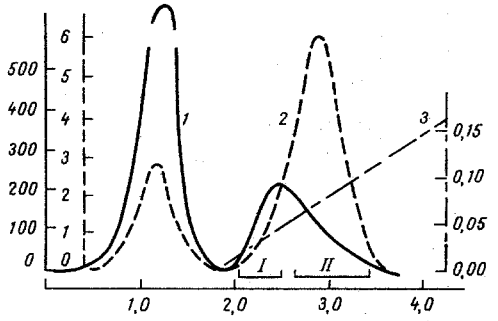

Fig. 1

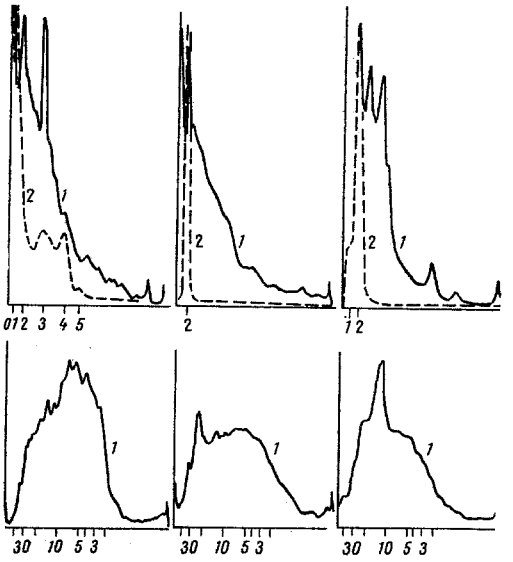

Fig. 2

Fig. 1. Chromatogram of fractionation of acid proteins of detergent extract of membranes on affinity adsorbent. I and II) Volumes of fractions I and II taken for further testing. Abscissa, volume of eluate (in liters); ordinate: 1) protein concentration (in μg/ml eluate), 2) ChE activity (in μmoles ATCh hydrolyzed in 1 min by 1 ml of eluate; units), 3) concentration of sodium chloride (in M).

Fig. 2. Electrophoresis of proteins of detergent extract of membranes, fraction I, and fraction II (from left to right): 1) protein profile; 2) ChE enzyme profile. Top row shows analysis by disk electrophoresis. Abscissa, mobilities of ChE isozymes (numbered in order of increasing mobility); bottom row – analysis by electrophoresis with SDS. Abscissa, mol. wt. ($\times 10^{-4}$ daltons).

of affinity adsorbent (3 × 50 cm). Desorption of the bound protein was carried out with a linear sodium chloride gradient in buffer. The resulting fractions were concentrated on small columns of DEAE-cellulose or DEAE-Sephadex.

The affinity adsorbent was synthesized by the method of Schmidt and Raftery [9]. The $[NH_2(CH_2)_5CONH-(CH_2)_3N^+CH_3)_3Br \cdot H\,Br$ ligand was "cross-linked" with 2% granulated agarose. For this purpose the agarose was activated with BrCN (25 mg/ml of gel). The ligand was added (0.24 mg to 1 ml of gel) to the washed activated agarose and the resulting suspension was left on a magnetic mixer for 2 days at 4-6°C. The gel was then thoroughly washed and equilibrated with buffer.

Electrophoretic investigations of the resulting protein preparations were carried out in polyacrylamide gel (PAG) by disk electrophoresis [3] and electrophoresis with sodium dodecyl sulfate (SDS) [11]. To determine the molecular weight of the protein by electrophoresis with SDS a calibration curve was plotted for human serum albumin and pancreatic ribonuclease, cross-linked with glutaraldehyde. After disk electrophoresis the cholinesterase (ChE) isozymes were detected by incubation of the PAG in 0.2 M Na-maleate buffer, pH 6.0, with $1.3 \cdot 10^{-3}$ M acetylthiocholine (ATCh) sulfate, $5 \cdot 10^{-3}$ M $CuSO_4$, and $5 \cdot 10^{-3}$ M $MgCl_2$ at 37°C. The densitograms of the gels were recorded by the IFO-451 microdensitometer. Protein was determined by Lowry's method and ChE activity by the method of Ellman et al. with dithionitrobenzoic acid and ATCh-iodide [4]. For photometry of the samples the SF-402 instrument (Perkin-Elmer, England) was used.

The rabbits were immunized as follows. To 0.6 ml of a solution containing not less than 0.1 mg of protein 0.4 ml of Freund's complete adjuvant was added. The resulting emulsion was injected subcutaneously at 10 points on the right side of the spine (0.1 ml at each point) and into the left thigh (0.5 ml). A second immunization was given in the same way 7-10 days later: The antigen was injected into the right thigh and on the left side of the spine. After another 7-10 days the animals were immunized a third time by injection of 0.5 ml of the emulsion into both thighs.

In the course of the experiments more than 2 kg of bovine caudate nuclei were used and 4 experiments carried out to isolate N-ChR. Altogether 10 rabbits were immunized, 6 with fraction I enriched with N-ChR and 4 with fraction II, containing higher ChE activity.

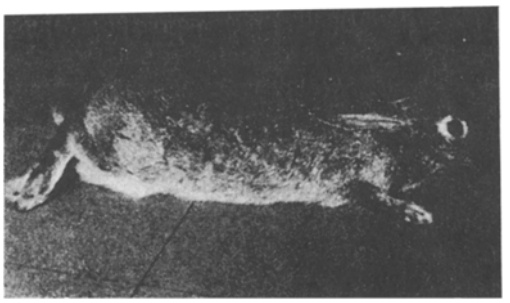

Fig. 3. Rabbit 1 week after last injection of protein of fraction I.

EXPERIMENTAL RESULTS

During isolation of the membranes from the caudate nuclei large quantities of soluble and weakly bound membrane proteins were extracted. Calculated per 100 g wet weight of tissue, about 0.5 g protein passed into the detergent extracts. Approximately 30% of the protein of the detergent extract was retained on the column of the anion-exchange resin and was subsequently eluted. The results of fractionation of this fraction (1 g protein) on the affinity adsorbent are given in Fig. 1. The yield of protein (after concentration) was 4 mg in fraction I and 5 mg in fraction II; the specific ChE activity in them was 60 and 230 units/mg protein respectively. ChE activity in the extract and eluate was found to decrease by about 50% over a period of 2 days. Electrophoretic analysis of the fractions is illustrated in Fig. 2.

In 3 of the 6 rabbits injected with protein of fraction I very severe myasthenia developed during the month after the first immunization (Fig. 3). Treatment of the myasthenia gravis by an anti-ChE preparation (0.25 ml of a 0.05% solution of neostigmine, intramuscularly) alleviated the animal's condition a little, but only temporarily: 2 rabbits died from respiratory arrest and 1 was killed in a very serious condition. No pathomorphological changes were found in the animals at autopsy. Proteins of fraction II caused no visible changes in the behavior of 4 rabbits during the 2 months after the first injection of the preparation.

It has frequently been shown that N-ChR is extracted from cholinergic receptor membranes by means of a detergent. As an acid protein, N-ChR is adsorbed on anion-exchange resins and is specifically bound by affinity adsorbents containing a quaternary nitrogen atom [7]. The protein fraction I now obtained possessed the same properties (Fig. 1). Like N-ChR from the electric organ of the electric eel [9], these proteins also were eluted from the affinity absorbent before the main mass of ChE (Fig. 1).

On the basis of similarity between the proteins of fraction I obtained from bovine caudate nuclei and N-ChR from the electric organ of fishes with respect to a number of physicochemical properties and their ability to induce myasthenia in rabbits immunized with them, it can thus be postulated that this fraction has a high content of N-ChR. Most probably the N-ChR of the electric organ of fishes and the N-ChR of the caudate nuclei have antigenic determinants of similar structure. The proteins of fraction I evidently cannot pass through the blood-brain barrier. Antibodies likewise are known not to penetrate into the brain. It accordingly seems possible to explain the myasthenia observed in the rabbit and causing their death by blocking of the N-ChR of the neuromuscular junction by antibodies produced against N-ChR of the caudate nuclei. Support for the validity of this interpretation of the phenomenon is given by the definite therapeutic effect of the anti-ChE preparation.

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DISCOVERY OF A STABLE CELL LINE ANTIGEN IN HUMAN INTERNAL ORGANS

I. N. Kryukova and T. S. Bobrova

UDC 616-006-092.9-008.9-097.5

A cell membrane antigen was found in stable cell lines of epithelial origin, identical to an antigen in homogenates of the human gastric mucosa. The antigen was detected by means of antiserum against extracted membrane antigens of HeP-2 cells, absorbed by a mixture of homogenates of human lung, liver, and papillomas of the human larynx and breast. The antigen described was found in some of the adenocarcinomas of the stomach that were tested. It differs from Gold's carcinoembryonic antigen and from the secretory component of IgA, it is not the structural antigen of the D-type primate oncornavirus produced by HeP-2 cells and, evidently, it is not coded by the genome of that virus.

KEY WORDS: stable human cell lines; cell membrane antigen; carcinoma of the stomach; gastric mucosa

For a number of years the writers have been engaged on research into the virion structural antigens of D-type primate oncornaviruses and the cellular antigens induced by these viruses in human tumors. In this connection the need arose for extraction of the membrane antigens of HeP-2 cells producing D-type oncornavirus (HeP-2b) [2, 6] and for the preparation of an antiserum against it.

In the present investigation, by means of the test system thus obtained, antigens of a number of stable human cell lines and of the gastric mucosa were investigated.

EXPERIMENTAL METHOD

Antigens. Solubilization and extraction of the cell membrane antigens by treatment with 3M KCl solution were described previously [8]. By this method antigens were extracted from stable human cell lines (HCL) HeP-2 (carcinoma of the larynx) and E16-b (carcinoma of the breast) [9], and also from primary human tumors: carcinomas of the kidney, tongue, stomach, three types of carcinoma of the breast, nodular mastopathy, and myoma of the uterus. The protein concentration in the preparations was determined by Lowry's method and they were concentrated with Lifogel to bring the proteins up to 1.55-1.7 mg/ml.

Homogenates also were prepared in Hanks' solution from stable HCL Fl (embryonic skin), ChET (human fibroblasts transformed by OB40 virus), ChET Rous (fibroblasts transformed by Rous virus), a suspension culture of Burkitt's lymphoma (the last three were obtained from G. I. Deichman), MB-157 and SH3 (carcinoma of the breast obtained from Texas Univeristy and Cancer Institute, Houston, USA), ChET + MPMV (human fibroblasts infected with Meson-Pfizer virus in the writers' laboratory), and also from numerous human primary tumors and normal tissues, and clarified by centrifugation at 9000 rpm.

Immune sera were obtained in rabbits by immunization into a lymph node by the method described previously [1]. Serum against the extracted membrane antigen of HeP-2 cells was obtained by the writers and designated AS against HeP-2 cell. Antiserum against twice purified and destroyed HeP-2b was provided by K. V. Il'in and designated AS against HeP-2b. It reveals the basic polypeptide of the nucleoid membrane, p27, of D-type oncornaviruses [6].

Department of Etiology and Diagnosis of Tumors, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR G. V. Vygodchikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 84, No. 12, pp. 690-693, December, 1977. Original article submitted March 14, 1977.

 0007-4888/77/8412-1728$07.50