MONOCLONAL ANTIBODIES TO INDIVIDUAL ANTIGENS OF THE NERVOUS SYSTEM OF *Helix pomatia*

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Investigations over many years, aimed at identification, isolation, and analysis of the physiological role of brain-specific proteins (BSP) in the basic manifestations of nervous activity and of pathology of the nervous system, have led to the formulation of various hypotheses, defining the place and functional significance of these nerve tissue components in brain activity [3]. In the first place, these studies demonstrated the antigenic heterogeneity of neuronal structures of varied degrees of complexity, defined for BSP the role of markers, of individual cell types and of functional structures of the cell (synapses, axons, cytoskeletal elements). The use of monoclonal antibodies not only broadened the spectrum of BSP-markers, but also led to the discovery of antigens characteristic of particular stages of cell differentiation, antigens identifying neurons, arising from the neural tube or neural crest, and also antigens specific for functionally combined groups of neurons, which demonstrated that monoclonal antibodies (MCA) may be used to analyze the architecture of nerve nets responsible for completed behavioral acts [2].

This paper describes the obtaining of monoclonal antibodies to nerve tissue antigens of the snail *Helix pomatia.* The structure and basic functions of its nervous system have been studied in sufficient detail, neuronal nets and their elements designed for performing particular actions — fixed and acquired — have been identified. As a result, the antibody preparations obtained may be used in the future to study the physiological role of the identified antigens and their involvement in the molecular mechanisms of behavioral acts.

EXPERIMENTAL METHOD

The test object was the nervous system of the snail *Helix pomatia.* As antigen for immunization we used an aqueous extract of the circumesophageal ring of nerve ganglia. The ganglia were freed from connective-tissue membranes, homogenized in deionized water (1:3 w/v), frozen and thawed three times, and centrifuged for 60 min at 2000*g* and 4°C. BALB/c mice were immunized intraperitoneally with the resulting aqueous extract three times at intervals of 7 days, with repetition of the same cycle 3 weeks later. During each injection the animal was given 100 μg of total protein. The first injection was given with Freund's complete adjuvant, subsequent injections with Freund's incomplete adjuvant (Sigma, USA). An intraperitoneal injection of 100 μg protein without adjuvant was given 4 days before hybridization.

Hybridization and subsequent cloning were carried out by the method of Köller and Milstein [5] and of Løvborg [6], using splenocytes of immunized mice and myeloma NSO cells for fusion. The cells were hybridized with the aid of polyethylene-glycol 1500 (Serva, West Germany). To screen the hybridomas, immunuenzyme dot analysis [4] on Millipore nitrocellulose filters (USA) was used. An aqueous extract of ganglia, freed from connective-tissue membranes, was used as the antigen; 1 μl of extract with a total protein concentration of 300 μg/ml or aqueous extracts of heterologous organs (liver, kidney, muscle, hemolymph) in the same concentration, being applied. The type of immunoglobulin was determined with the aid of standard antibodies to different types of mouse immunoglobulin (Sigma, USA) and the content of immunoglobulin in the culture fluid was determined by solid-phase radioimmunoassay using ^{125}I-labeled protein A from *Staphylococcus aureus* [8], unconcentrated culture medium and the same medium concentrated tenfold.

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TABLE 1. Immunocytochemical Localization of MCA in Nerve Ganglia of *Helix pomatia*

Clone, subclone	Parietal ganglion	Visceral ganglion	Pool D	Neural ganglion	Pedal ganglion			Cerebral ganglion		
					ventral part	dorsal part	med. part	meta	meso	proto
1C5	—	++	—	—	++	++	+	—	—	—
1C5-D9	—	++	—	—	++	++	+	—	—	—
6B4	++	++	—	+	—	—	—	++ Mtc-2 Mtc -3	—	—
6B4-G11	++	++	—	—	—	—	—	Mtc -2 Mtc -3	—	—
4B2	—	++	—	—	++	—	—	+	—	—
4B1	—	++	—	+	—	++	—	++	—	—
4B1-E4	—	++	—	—	—	++	—	++	—	—
4B5	—	—	—	—	+	—	—	—	—	—
4B5-D8	—	—	—	—	+	—	—	—	—	—

Immunohistochemical analysis was carried out on cryostat sections of freshly frozen circumesophageal ganglionic ring of *Helix pomatia,* using medium conditioned by hybridomas and antibodies to mouse immunoglobulin, conjugated with horseradish peroxidase (Sigma, USA), in dilutions of 1:500 and 1:250 [7]. The peroxidase reaction was revealed by the aid of 3,3′-diaminobenzidine (Serva, West Germany) and the cell nuclei were weakly counterstained with hematoxylin. Neurons in ganglia of the circumesophageal ring were identified in accordance with the classification of Maksimova and Balaban [1].

EXPERIMENTAL RESULTS

Of the 103 primary hybridomas obtained, we chose seven whose culture fluid gave a positive reaction on enzyme immunoassay with nerve tissue antigens of *H. pomatia,* but did not react with extracts of muscle and internal organs of *H. pomatia.* On further subcloning by the limiting dilutions method, 17 positive subclones were isolated, whose culture fluid was used for immunohistochemical investigations. The following MCA were analyzed in detail: IC5 (IC5-D9):IgC_{2a} (0.37 μg/ml); 6B4 (6B4-G11):IgG_{2a} (0.35 μg/ml); 4B5 (4B5-D8):IgM (0.2 μg/ml); 4B1 (4B1-E4):IgG_1 (0.22 μg/ml); 4B2:IgG_2 (0.17 μg/ml).

The culture fluid of clone 8B5:IGG_1 (0.35 μg/ml), which did not give a positive reaction with nerve tissue antigens of *H. pomatia,* was used as the control to the immunohistochemical experiments.

The results of immunohistochemical analysis are given in Table 1. MCA of clone IC5 and its subclone IC5-D9 were located in the cytoplasm of neurons of the visceral ganglion, including motoneurons closing and opening the pneumostone, and also in cells of the ventral and dorsal parts of the pedal ganglion. MCA of clone 6B4 and subclone GB4-G11 were discovered in the cytoplasm of metacerebral motoneurons Mtc-2 and Mtc-3 (a motoneuron controlling retraction of the eye tentacle), and of command neurons of defensive behavior LPa3 and LPa5; the reaction developed less strongly in the cytoplasm of RPa2, RPa3, and neurons with a burst type of activity, namely RPa5-RPa9.

MCA of clone 4B5 and its subclone 4B5-D8 reacted with cytoplasmic antigens of the neurons only in the ventral part of the pedal ganglion, whereas MCA of clone 4B2 reacted with antigens of neurons of the visceral ganglion, including motoneurons opening and closing the pneumostome, giant neurons of the pedal ganglion, and small peripheral neurons of the metacerebrum.

MCA of clones 4B1 and 4B1-E4 interacted with antigens localized in the cytoplasm of the modulator command neuron of feeding behavior Mtc-1 and motoneuron Mtc-2, and in neurons of the visceral ganglion, including motoneurons opening and closing the pneumostome.

We thus obtained MCA reacting selectively with individual neuron pools located in ganglia forming the circumesophageal ring of the central nervous system of *Helix pomatia* and performing various functions in the course of formation of complex forms of behavior.

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ENZYMIC INDICATOR OF IMMUNOBIOLOGICAL ACTIVITY OF ANTIGENS

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A widely used indicator of the immunologic properties of different antigens, based on analysis of the serum antibody titer, is general in character and reflects only the end result of the immune process. Such an approach, if used during the production of vaccine preparations, cannot completely reflect all the diversity of immune reactions taking place in man and animals. At the present stage of development of immunology, the study of the action of different antigens on individual components of the immune system is very important. Accordingly, the development of a test system which would allow antigens used for vaccine production to be screened on separate populations of immunocompetent cells is an urgent task. An important aspect of this problem is the macrophagal component of the immune system. We know that an antigen undergoes processing in the macrophages, after which it is presented to lymphocytes [5]. In turn, when interacting with the antigen, the macrophage becomes activated, and this can be revealed by a number of methods based on determination of the respiratory burst, intensification of phagocytic and migration functions, and incorporation of fluorescent substances [4, 7]. However, these methods do not completely reflect the trigger function of antigens.

The activated macrophage accumulates and excretes a whole range of substances, including hydrolytic enzymes [4, 7, 8]. One such enzyme is acid phosphatase, activity of which is modified after stimulation of the macrophage and it can be used as an indicator of its activation [4].

The aim of this investigation was to develop a test system whereby the degree of activating ability of antigens of different nature in a culture of mouse peritoneal macrophages can he determined biochemically.

EXPERIMENTAL METHOD

To obtain peritoneal macrophages CBA mice aged 5-6 weeks were used. The mice were given an intraperitoneal injection of 5 ml of 3% peptone. On the 3rd day cells of the peritoneal exudate were harvested by flushing out the peritoneal cavity with 5 ml of medium 199, pH 7.2, containing fetal calf serum (5%), glutamine (0.03%), heparin (5 IU/ml), and antibiotics (penicillin and streptomycin, 100 IU/ml and 100 μg/ml respectively). A firm monolayer of macrophages was obtained by incubating $(3.5\text{-}4.0) \cdot 10^6$ peritoneal cells in 1.5 ml of medium for 2 h in 40-ml plastic Petri dishes at 37°C. After removal of the medium the adherent cells were washed twice with culture medium (without heparin, but with the addition of HEPES), and incubated in that medium after addition of the antigen (experimental samples) or without it (control samples) for 24 h in an atmosphere containing 5% CO_2, at 37°C. At the end of the incubation period the culture medium was removed, and the cell monolayer was washed once with Hanks' salt medium, containing 10% bovine serum, and then with buffered 0.15 M NaCl solution, pH 7.2. The

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