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Polyethylene Glycol-Conjugated Immunoliposomes Specific for Olfactory Ensheathing Glial Cells

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A novel immunoliposome delivery system was developed for directed transport into cultured olfactory epithelium cells. Monoclonal antibodies against glial fibrillary acidic protein (GFAP) served as a vector. Fluorescence microscopy showed that the target cells are specifically stained with Dil dye incorporated into liposomal membranes. This transport system holds promise for the delivery of bioactive substances to olfactory epithelial cells and modulation of their capacity to stimulate axonal regeneration.

Key Words: *immunoliposome; olfactory epithelial cells; glial fibrillary acidic protein; directed transport*

Much recent attention is paid to cell therapy for spinal cord injuries [4,5,7,10-13,15]. Schwann cells [13], stromal stem cells [7,12,15], and mesenchymal stem cells of the bone marrow [4,5,11] were used to stimulate regeneration and remyelinization in rats with experimental trauma and ischemia of the spinal cord.

Experimental studies performed from the 1990s to the present time revealed a new source of cells for transplantation in spinal cord injury. They are presented by olfactory ensheathing cells (OEC) present in olfactory epithelium, olfactory nerve, and surface layers of the olfactory bulb.

Published data indicate that the positive effects of OEC transplantation are determined by the optimal microenvironmental conditions for the growth and myelinization of regenerating axons and their ability to suppress astrocyte activation and glial scar formation, stimulate the growth of blood vessels, and provide migration of Schwann cells to the site of damage [14]. It is necessary to develop a novel specifically-directed transport system to deliver bioactive substances to OEC and modulate their capacity to stimulate axonal regeneration.

The aim of this work was to create polyethylene glycol (PEG)-conjugated immunoliposomal systems specifically binding to OEC. Monoclonal antibodies against glial fibrillary acidic protein (GFAP) served as the vector for these systems. Antibodies were previously synthesized in our laboratory and specifically bind to these cells.

MATERIALS AND METHODS

Liposomes were prepared using previously described method (modified according to the purpose of our study) [6].

The following lipid components were used for liposomes: egg yolk lecithin; cholesterol; distearoylphosphatidylethanolamine (DSPE) conjugated with

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PEG (molecular weight 2000 Da, PEG-2000); and maleimido-4-(*p*-phenylbutyryl)-phosphatidylethanolamine (MBP-PE) for covalent binding to the protein (all lipid components were from Avanti Polar Lipids). Other reagents, solvents, and adjuvants were of maximum chemical purity. All manipulations except for short-lasting procedures, including weighing and centrifugation, were performed in high-purity argon atmosphere. The weighted samples of lecithin, cholesterol, MBP-PE, DSPE-PEG, and fluorescent dye Dil (molar ratio 23:16:1:1.6:0.4) were dissolved in a chloroform-methanol mixture (9:1, 10 mg total lipids per ml solvent). The mixture was dried in a rotor evaporator under low pressure. Dry lipid film was dissolved in absolute cyclohexane, frozen in liquid nitrogen, and lyophilized. The mixture of lipids was emulsified in 0.1 M phosphate buffered saline. The hydrated emulsion was sonicated in a G112SP1 Special Ultrasonic Cleaner (Laboratory Supplies Co) to prepare liposomes and passed 15 times through polycarbonate membrane filters (pore size 400, 200, 100, and 50 nm) using a mini-extruder (Avanti Polar Lipids, Alabaster).

Monoclonal antibodies against GFAP served as the specific vector. Monoclonal cells producing anti-GFAP antibodies were obtained by the standard method [8] with modifications [3]. PEG-DMSO was used as a coupling agent (Sigma). After isolation of the producer clone, hybrid cells were injected intraperitoneally to BALB/c female mice to cause ascites. Monoclonal anti-GFAP antibodies were isolated from the ascitic fluid using immunosorbents of CNBr-Sepharose [2].

Liposomes were conjugated to thiolated antibodies by the maleimide method [9]. Activity of antibodies was tested immunohistochemically at all stages of thiolation and conjugation.

The preparation of immunoliposomes was tested in a reaction of specific binding to fixed cultured OEC from the olfactory epithelium of 10-13-day-old rats. The olfactory epithelium and *lamina propria* were isolated from the superior nasal passage, cut into fragments, and dissociated with 0.1% trypsin for 15 min. Enzyme activity was inhibited by adding culture medium with 5% fetal bovine serum (FBS). Mechanical dissociation was performed using a Pasteur pipette. After centrifugation, the pellet was resuspended in 4 ml growth medium (DMEM/F12, 10% FBS, L-glutamine, and penicillin/streptomycin) and grown for 10-14 days in culture dishes (Costar). The medium was replaced at 3-day intervals. For testing immunoliposome preparations, cultured cells were treated with 0.1% trypsin for 5 min, centrifuged, put on poly-L-lysine-coated coverslips (22×22 mm), and grown for 3-5 days [1].

For more precise verification of cultures, immunoperoxidase reaction with monoclonal anti-GFAP antibodies (primary antibodies) and peroxidase-labeled biotinylated goat antibodies against mouse Ig (secondary antibodies, Vector Laboratories) was used.

The culture was fixed with ice-cold methanol, washed 3 times with phosphate-buffered saline (FBS, 10-min washing), and incubated with PEG-conjugated immunoliposomes (1 ml) for 3 and 6 h. The cultures were than washed 5 times with PBS (Gibco) and visualized. The appearance of specific fluorescence due to the introduction of a fluorescent lipid dye (Dil) into the liposomal membrane was recorded using a Leica DM LB HC microscope (light microscopy and fluorescence at ×400, filters 515-560-nm).

Quantitative study of total phospholipids in aqueous emulsions (preparation of immunoliposomes) [2] was based on the spectrophotometric measurement of colored complex compounds, which consisted of lipid molecules and iron (III) thiocyanate (rhodanide). The calibration curve was constructed from phosphatidylcholine solutions in various concentrations.

Total protein content was measured in the reaction with bicinchoninic acid [3] using BSA Protein Assay Reagent Kit 23227 (Pierce). The sample (preparation of immunoliposomes, 0.1 ml) was mixed with 2 ml working solution and incubated at 37°C for 30 min. Photometry was performed at 562 nm. The calibration curve was constructed according to dilution of the standard BSA solution.

RESULTS

The amount of conjugated antibodies was 150 μg per 1 μmol phospholipids, which is consistent with published data on immunoliposome preparation [9]. The theoretical calculations were performed taking into account that liposomes are unilamellar vesicles with a diameter of 70 nm. Each liposome consists of 100,000 lipid molecules. Hence, one liposome contains 60 molecules of conjugated antibodies at a specified phospholipid-protein ratio in the sample. These data are consistent with typical characteristics of liposomes.

Immunohistochemical study revealed high affinity and suitability of anti-GFAP antibodies for these systems.

Vector function of PEG-immunoliposomes was confirmed in *in vitro* experiments. Fluorescence microscopy with 515-560-nm filters showed that fixed OEC culture exhibits strong fluorescence after incubation in the presence of PEG-immunolipo-

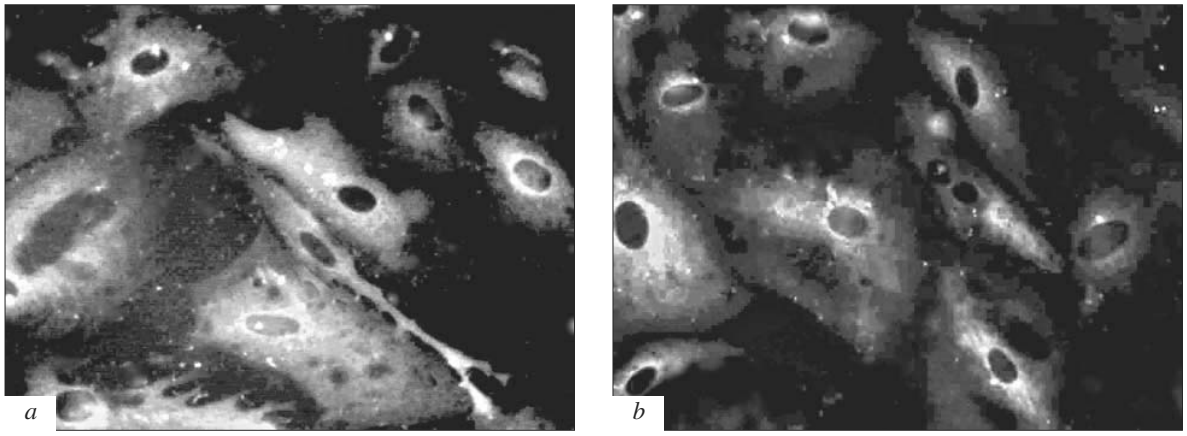

Fig. 1. Microphotographs of cultured rat OEC (DiI staining, ×400). Incubation with PEG-immunoliposomes containing anti-GFAP antibodies as the vector for 3 (*a*) and 6 h (*b*).

somes conjugated with anti-GFAP antibodies (vector, Fig. 1).

It should be emphasized that these features are not observed under the following control conditions:

1) incubation with PEG-liposomes labeled with DiI and not conjugated to proteins;
2) incubation with PEG-liposomes labeled with DiI and conjugated to nonspecific mouse Ig;
3) preincubation with free anti-GFAP antibodies in the absence of liposomes, and subsequent incubation with PEG-immunoliposomes labeled with DiI and containing anti-GFAP antibodies as a vector; and
4) incubation of cultured fibroblasts with PEG-immunoliposomes labeled with DiI and containing anti-GFAP antibodies as a vector.

These data indicate that the preparation of PEG-immunoliposomes (vector-oriented by anti-GFAP antibodies) retains specificity of conjugated antibodies and specifically interacts with GFAP, a specific marker of OEC.

Hence, conjugation of monoclonal anti-GFAP antibodies to PEG-liposomes results in the formation of a high-specificity delivery system for directed transport of diagnostic and medicinal drugs to these cells.

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