EFFECT OF MONOCLONAL ANTIBODIES TO BOVINE NERVE GROWTH FACTOR (NGF) ON NGF-INDUCED CELL DIFFERENTIATION IN VITRO

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Nerve growth factor (NGF) is a protein which maintains the vital activity of sympathetic and sensory neurons and is involved in the differentiation of various tissues, rising from cells of the neural crest [3]. Cells of certain neurogenic tumors, such as neuroblastomas, pheochromocytomas, and melanomas [2, 4, 9] preserve receptors for NGF on their surface, indicating that NGF may have a role in regulation of the development of these tumors.

NGF is present in large quantities in the submandibular salivary glands of mice [1], in snake venoms [7], and in bovine seminal plasma [5]. The traditional test object is murine NGF, which is secreted in the form of a multimolecular complex, consisting of three subunits (α, β, γ). NGF from bovine seminal plasma, unlike murine NGF, contains only the functionally significant β-subunit [6]. In its primary structure it possesses 93% homology with human NGF [13] and it has biological activity similar to that of murine NGF [6].

The aim of this investigation was to obtain monoclonal antibodies (McAb) to bovine NGF and to study their effect on NGF-induced differentiation of PC-12 cells and spinal ganglion cells of chick embryos – the most widely used objects for the study of this process [4].

EXPERIMENTAL METHOD

NGF was isolated from bovine seminal plasma by a modified method in [5]. After filtration of the seminal plasma through ponds with DEAE- and CM-Trisacryl (LKB, Sweden), connected in series, in 20 mM sodium-phosphte buffer, pH 6.8, fractions containing protein were acidified with 1M citric acid to pH 3.0, after which NaCl was added to a final concentration of 0.4 M. After application to SP-Sephadex C-25 ("Pharmacia," Sweden) the column was washed successively with 0.1 M sodium-citrate buffer, pH 3.0, containing 0.4 M NaCl, then with the same buffer without NaCl and, finally, 50 mM Tris-HCl buffer, pH 9. After washing the column with 50 mM Tris-HCl buffer with 0.1 M NaCl the NGF was eluted with the same buffer containing 0.3M NaCl. Fractions in which the purity of NGF exceeded 80% were pooled and rechromatographed on SP-Sephadex C-25, followed by elution of the NGF in a 0-0.5 M NaCl gradient. The resulting NGF preparation had a purity of over 98% as shown by SDS-electrophoresis and isoelectric focusing.

To obtain hybridomas synthesizing McAb to bovine NGF, BALB/c mice were immunized 3 times with the NGF Preparation (50 μg per mouse) at weekly intervals. The first immunization was carried out with Freund's complete adjuvant ("Difco," USA). Booster immunization was undertaken 3 days before splenocytes were taken for the fusion experiment. Myeloma cells of strain SP2/0-Ag14 were fused with immune splenocytes with the aid of polyethylene

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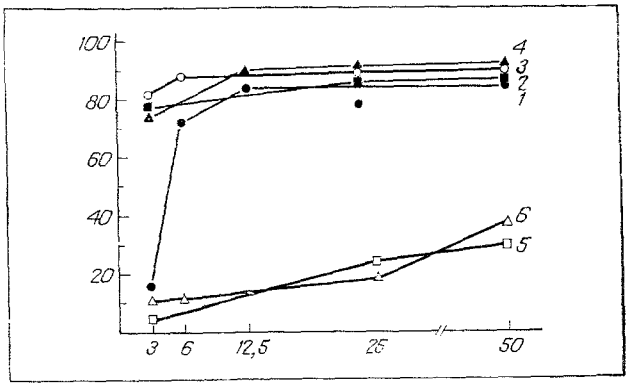

Fig. 1. Effect of McAb on NGF-dependent differentiation of PC12 cells. 1) NGF; 2-6) NGF with excess of McAb: 2) $1B_2$, 3) $1C_6$, 4) $2B_5$; 5) C_3-1-C_3; 6) $1C_3$. Abscissa, concentration of NGF (in ng/ml); ordinate, per cent of cells with primitive axons.

glycol, with mol. wt. of 4000 ("Serva," West Germany), by the usual method [8]. Hybrid clones were isolated on selective medium containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterine, and 1.6×10^{-5} M thymidine ("Sigma," USA). Producer clones of NGF antibodies were isolated by an immunoenzyme method [12].

Affinity purification of antibodies (AB) was done on NGF-sepharose Cl-4B [11] with a concentration of covalently bound protein of 1 mg/ml sepharose, Immunoglobulins were eluted from the column by 4M $MgCl_2$ or 10 mM HCl. The purified AB were dialyzed against a buffer solution containing 50 mM tris-HCl, pH 6.8, and 0.15 M NaCl. The class of McAb was determined by enzyme immunoassay using a conjugate based on horseradish peroxidase and anti-IgG or anti-IgM-antibodies ("Sigma").

The biological activity of NGF and the effect of McAb on it were studied on cells of rat pheochromocytoma PC12, obtained from Dr. M. Saarma (Institute of Chemical and Biological Physics, Academy of Sciences of the Estonian SSR). The cells were cultured on Eagle's medium in Dulbecco's modification ("Flow Laboratories," England) with the addition of 10% embryonic calf serum and 5% horse serum (Flow Laboratories). Spinal ganglia were obtained from 9-day chick embryos [10] (obtained from the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR).

Before addition of NGF and/or McAb the cultures were transferred to serum-free medium. The mixture of NGF and McAb was preincubated at 37°C for 1 h. PC12 cells with axons were counted after 48 h. For each NGF concentration two fields of vision, each containing 100 cells, were counted in three repetitions of the experiment. The results of the experiments on ganglia were read after 3 days.

EXPERIMENTAL RESULTS

The NGF used to immunize the mice was tested for biological activity. Differentiation of PC12 cells was observed 24 h after its addition to the culture: the cells began to form primitive axons, which became clearly visible after 48 h and remained for 7-12 days. In the presence of saturating concentrations of NGF (12 ng/ml) about 80% of cells of the population differentiated to form primitive axons. Differentiation of the sympathetic ganglia of the chick embryos began to appear 72 h after the addition of NGF. In the absence of NGF, no growth of primitive axons took place.

To obtain the maximal immune response during immunization of the mice several doses of NGF were tested. A single injection of 50 μg was found to be the optimal dose.

As a result of fusion of the immune splenocytes with SP-2/O Ag14 myeloma cells about 600 primary hybridoma clones were obtained. As a result of their screening, five clones synthesizing antibodies to NGF at a stable rate were selected. All antibodies were of the IgG class. Immunoblotting NGF of different degrees of purification with culture medium of each of the five clones revealed one band corresponding to a molecular weight of 13 kD.

The study of cells producing antibodies to NGF showed that the clones were mainly unstable with respect to this feature: despite the large amount on primary screening, after culture for 2 months McAb synthesis had been lost by most clones. The selected clones also differed in efficiency of their antibody formation during passage through mice; this sufficiency, moreover, did not correlate with antibody production during cell culture.

During chromatography on NGF-sepharose, the antibodies were successfully eluted both by 4 M $MgCl_2$ solution, pH 5, and by 10 mM HCl solution, pH 2. According to the results of SDS-electrophoresis and isoelectric focusing, affinity-purified antibodies were homogeneous in both cases.

Antibodies of five monoclonal producer cells were studied in biological tests. With respect to their ability to affect differentiation of PC12 cells these McAb can be divided into two groups (Fig. 1). Antibodies of group 1 ($1\text{-}C_3$ and $C_3\text{-}1\text{-}C_3$) blocked the stimulating action of NGF on PC12. McAb of group 2 ($1\text{-}B_2$, $1\text{-}C_6$, $2\text{-}B_5$) did not affect the response of the PC12 cells in the presence of high concentrations of NGF. In this case the number of cells with primitive axons was about 80% in both experiment and control (without antibodies). However, on the addition of small amounts of NGF (3 ng/ml) to the cells in the presence of an excess of group 2 antibodies, the number of cells with primitive axons exceeded that of cells stimulated by NGF without antibodies. Treatment of cells with McAb of this group but without NGF did not give rise to any morphological changes.

In experiments on spinal ganglia of chick embryos, McAb of group 1 blocked growth of primitive axons induced by NGF; McAb of group 2 had no effect on cell differentiation.

The McAb to bovine NGF thus obtained differed in their effect on NGF-dependent differentiation of target cells. Inhibition of differentiation of McAb of group 1 was probably due to the fact that the NGF-McAb complex was unable to bind with NGF receptors. The effect of potentiation of the biological action of NGF in low concentrations in the presence of group 2 McAb is interesting. This effect may perhaps be connected with the effect of antibodies on cluster formation by the NGF-receptor complex, as takes place, for example, in the case of epidermal growth factor [14]. The possibility likewise cannot be ruled out that the GRF-antibody complex has increased affinity for the NGF-receptor.

The study of the nature of stimulation of the differentiation process induced by NGF by McAb obtained as described above requires further investigation. However, McAb of this type can evidently be used as a new tool with which to study the mechanism of action of NGF.

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