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OBTAINING MONOCLONAL ANTIBODIES TO HUMAN GROWTH HORMONE AND THEIR CHARACTERISTICS

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Growth hormone or somatotrophin (STH) is a pituitary polypeptide hormone with molecular weight of 22 kD. This hormone is characterized by the diversity of its biological action and by its well-marked species-specificity. STH of animal origin has no growth effect in man and virtually does not give any cross reaction with antiserum to human STH.

The solution of certain scientific and practical problems requires quantitative determination of STH in biological fluids and tissue extracts. Until recently, polyclonal antibodies obtained from hyperimmunized animals have been used in diagnostic immunoassay systems. Nowadays, with the development of new and improvement of existing immunochemical methods of determination of human STH, as well as of other protein hormones, monoclonal antibodies (McAb) are beginning to be used on an increasing scale [3, 10], because of the stability of their properties and their high specificity. The use of McAb directed toward particular epitopes in the STH molecule enables reagents to be standardized and makes the process of creation of diagnostic systems technologically more refined.

The aim of this investigation was to obtain hybridomas producing McAb to human pituitary STH (STH_{pit}), and to study the properties of the McAb thus obtained and the possibility of using them in immunoassay systems.

EXPERIMENTAL METHOD

Highly purified preparations of human STH_{pit}, and also human prolactin (PRL), human placental lactogen (PL), bovine STH, bovine PRL, and porcine PRL, isolated in the Institute of Experimental Endocrinology and Hormone Chemistry, Academy of Medical Sciences of the USSR, by methods according to [14, 7, 5, 2], respectively, were used. As well as STH_{pit}, a biosynthetic preparation of human STH obtained by a genetic engineering method (STH_{bio}) in connection with the "Human Growth Hormone" Program of the Academy of Sciences of the USSR, the Ministry of the Medical and Microbiological Industry of the USSR, and the Ministry of Health of the USSR, also was used.

To obtain hybridomas, BALB/c mice were immunized with highly purified STH_{pit}. The course of immunization consisted of three injections of the preparation (100, 50, and 50 μg) in Freund's complete adjuvant with intervals of 4 weeks between injections. Before fusion

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of the cells the mice were reimmunized by a single injection of 100 μg of the hormone in 0.05M phosphate buffer (PB), pH 7.2. Hybridomas were obtained by fusing spleen cells of immunized mice with myeloma cells of the X-63.Ag8.653 strain by the method in [6]. Supernatants of the growing hybrid clones were tested for the presence of McAb to STH by direct enzyme immunoassay (EIA) by the method in [18], with a reaction volume of 60 μl per well. For the assay, STH_{pit} was fixed to flat-bottomed 96-well panels (Moscow Experimental Factory, All-Union Research Institute of Medical Technology) at 4°C in a concentration of 1 μg in 1 ml of 0.05M bicarbonate buffer. The conjugate of antibodies to mouse IgG_1 was obtained by the method in [17].

To obtain McAb in preparative amounts the hybridoma cells were grown in the form of ascites in the peritoneal cavity of BALB/c mice. The McAb were isolated from the ascites fluid by salting out with ammonium sulfate, followed by ion-exchange chromatography on DEAE-52 ("Whatman," England) in a gradient of 0.005-0.25 M sodium-phosphate buffer, pH 8.0 [8]. The concentration of McAb purified in this way was determined by measuring optical density at 280 nm [12].

The subclass of the McAb was established by the double immunodiffusion test, using antisera to subclasses of mouse immunoglobulin heavy chains ("Serotec," England). Electrophoresis of the purified McAb under reducing and nonreducing conditions also was carried out in polyacrylamide gel (PAG) in the presence of sodium dodecylsulfate (SDS) [15].

Epitopic specificity of McAb to STH1 and McAb to STH2 was established by ELISA after fixation of STH_{pit} on the panels. Panels with adsorbed STH_{pit} were treated with a solution of McAb to STH1 or McAb to STH2 in a concentration of 100 μg/ml, and after incubation for 1 h at 37°C the panels were washed with PB and a conjugate of McAb to STH2 with peroxidase (1.5 μg/ml) was added, the panels were again kept for 1 h at 37°C, after which they were thoroughly washed and the chronogenic substrate was added to the wells (orthophenylenediamine-HCl, "Serva," West Germany). Enzyme activity was then recorded at 492 nm. The conjugate of peroxidase with McAb to STH2 was synthesized by the periodate method [17].

Immunodetection of STH_{pit} in different dilutions was then carried out by means of ^{125}I-labeled McAb to STH2, at points on nitrocellulose filters [16] and by the immunoblotting method [4]. The McAb STH2 were iodinated with the ^{125}I isotope by means of chloramine T by the method in [9].

During determination of STH_{pit}, the noncompetitive variant of ELISA was used (reaction volume 100 μl per well). Monospecific polyclonal rabbit immunoglobulins against human STH (10 μg/ml), purified by the method in [11] on a conjugate of human STH and sepharose CNBr4B

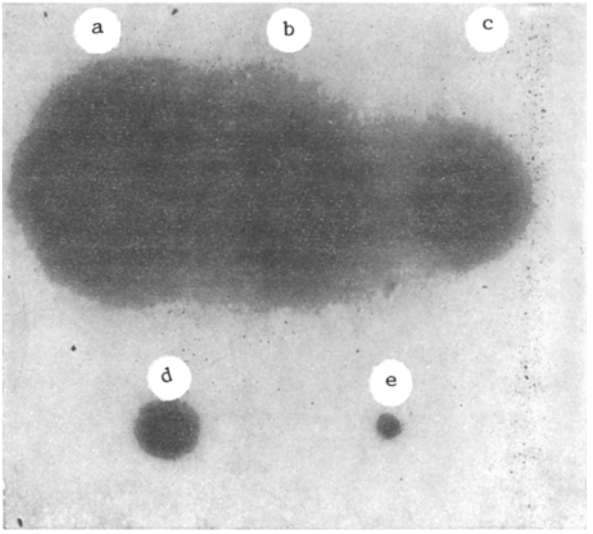

Fig. 1. Detection of STH_{pit} at points on nitrocellulose filters with the aid of ^{125}I-labeled McAb to STH2. Concentration of STH_{pit} (in ng) per point: a) 1000, b) 500, c) 100, d) 10, e) 1.

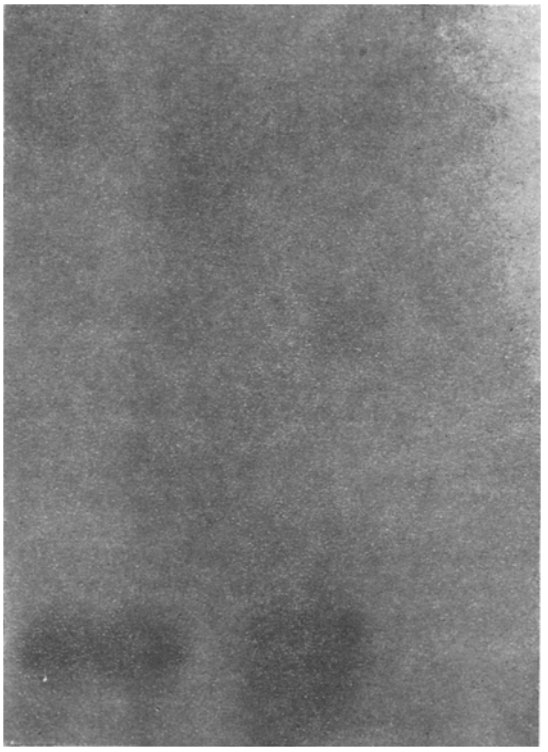

Fig. 2. Radioimmunoblotting of STH_{pit} (development with ^{125}I-labeled McAb to STH2). Quantity of STH_{pit} (in ng) added to gel: a) 150, b) 250, c) 500.

("Pharmacia," Sweden), were adsorbed on 96-well panels at pH 9.6. The panels covered with antibodies were treated with a solution of antigen in the necessary dilution in PB, containing 0.15 M NaCl, 0.1% Tween-20, and 0.2% bovine serum albumin. The samples was incubated for 2 h at 37°C and the panels washed with PB containing Tween-20. In the 3rd stage, 100 µl of the conjugate of McAb to STH2 with peroxidase was added to each well in the working dilution. After incubation for 1 h at 37°C enzyme activity was determined by measuring the optical density of the reaction product in the wells at 492 nm.

EXPERIMENTAL RESULTS

During hybridization of spleen cells of a hyperimmune mouse (the antibody titer to STH_{pit} in EIA was 1:25,600) with the myeloma cell line X-63.Ag8.653, growth of hybrid cells was found in 64% of the wells. In eight wells a positive reaction was obtained in the indirect EIA for STH. After cloning of the hybridomas, two clones producing McAb to human STH were obtained from two wells by the limiting dilutions method: McAb to STH1 and McAB to STH2. Both variants of McAb belonged to the IgG1 isotype and gave one band on PAG electrophoresis under nonreducing conditions, corresponding to proteins with mol. wt. of 160 kD, whereas under reducing conditions they gave two bands, one corresponding to proteins with mol. wt. of 50 kD (heavy chain) and the other, to mol. wt. of 25 kD (light chain).

The specificity of the McAb to STH1 and McAb to STH2 was tested by the indirect EIA method for ability to bind the homologous STH_{pit} or STH_{bio} antigen, and also bovine STH, human PL, human PRL, bovine PRL, and porcine PRL. The choice of these antigens was dictated by the presence of species specificity of STH and also by structural homology of STH, PL, and PRL. McAb to STH1 and McAb to STH2 reacted only with human STH and not with other structurally related antigens, evidence of the high specificity of the McAb obtained. It was shown by EIA that McAb to STH1 and McAb to STH2 are directed most probably against a common antigenic region, i.e., they do not differ in their epitopic specificity.

Human STH is characterized by molecular polymorphism. Structural variants of the hormone differing in biological and immunologic activity, and the relation between them, are found in the pituitary gland [13]. Highly purified preparations STH_{pit}, homogeneous with respect

to their N-terminal amino acid, during PAG-electrophoresis therefore usually give not only the dominant component (mol. wt. 22 kD), but also other electrophoretic components.

According to our data [1], STH_{bio} obtained in the USSR by a genetic engineering method, and using the present investigation, is a multicomponent product, and in its physicochemical, biological, and immunologic properties corresponds in the radioimmunoassay system for STH_{pit} to the dominant variant of STH_{pit}.

Since the titer of McAb to STH2 in the ascites fluid (1:206,000) was significantly higher than the titer of McAb to STH1, McAb to STH2 were investigated in more detail.

The study of binding of McAb to STH2 with STH_{pit} and STH_{bio} in EIA showed that McAb to STH2 possess about equal immunoreactivity to these antigens. This may be evidence that McAb to STH2 interact with an antigenic determinant belonging to the dominant monomeric component of human STH (22 kD). Under these same conditions McAb to STH2 did not react with human PL, which possesses a high degree of structural similarity (85%) with human STH [19]. The absence of reaction with the structurally related human PL may be evidence that the epitope recognized by the antibodies produced is unique for human STH.

Affinity of McAb to STH2 for STH_{pit} was tested by the dot blotting method on nitrocellulose filters and in an immunoblotting system with the aid of ^{125}I-labeled McAb to STH2. By the dot blotting method it was possible to detect 1 ng of STH_{pit} (Fig. 1), evidence of a high degree of affinity of the McAb to STH2.

The results of radioimmunoblotting are shown in Fig. 2. It will be clear that in the immunoblotting system, different numbers of immunoreactive components of the hormone could be found depending on the quantity of added STH_{pit} by the aid of ^{125}I-McAb to STH2. If 150 ng of STH_{pit} (A) was added to the gel, one immunoreactive component corresponding to the monomeric form of the hormone was found. An increase in the amount of STH_{pit} enabled a component corresponding to the dimer form of the hormone to be identified, as well as an immunoreactive component with smaller molecular weight than that of the monomer (B).

According to our data, McAb to STH1 and McAb to STH2 most probably have identical epitopic specificity, and for that reason they could not be used in one system for determining human STH by solid-phase EIA in the "sandwich" version. When an attempt was made to use McAb to STH2 as fixing antibodies, they were found to have low adsorptive ability relative to polystyrene panels. For quantitative evaluation of STH_{pit} in dilutions from 1 to 200 ng/ml PB, the following version of the "sandwich" system was therefore chosen: monospecific affinity-purified antibodies to STH_{pit} were fixed to the panels in a concentration of 10 μg in 1 ml of 20 mM bicarbonte buffer, and the panels were then incubated with different dilutions of STH_{pit}, using a conjugate of McAb to STH2 with horseradish peroxidase to identify the antigen. The range of quantitative determination in such a system was 12-200 ng/ml.

McAb with high specificity to human STH were thus obtained and characterized in the course of this investigation. The McAb produced can be used to detect human STH by immunochemical methods in fractions during isolation of the hormone both from pituitary glands and when human STH is obtained by genetic engineering methods.

Because of the possibility of producing large quantities of McAb to STH2 with particular immunologic characteristics with the aid of a hybridoma technique, immunosorbents for use in optimizing the process of isolation of the hormone can be created on their basis.

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RYODIPINE – A NEW FLUORESCENT PROBE FOR DETECTING DIFFERENCES BETWEEN LYMPHOCYTES

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Several years ago a fluorescent method was suggested for distinguishing between lymphocyte populations [7, 9, 14]. This method was based on the use of the fluorescent probe MBA (3-methoxybenzanthrone), on staining with which B lymphocytes fluoresce more brightly than T lymphocytes. Later, diagnostic tests have been created on the same principle, by which it is possible to detect bronchial asthma [5, 8] and some malignant blood diseases [1, 6]. In these cases the diagnosis is based on recording the intensity of fluorescence (F) of single leukocytes in human blood, stained by the MBA probe, and comparing histograms of distribution of the cells with respect to F in normal subjects and in the pathological state.

The further development of the technique of fluorescent probing of cells necessitates the search for new fluorescent probes. The investigation described below is one step in this direction. It demonstrates that the fluorescent hypotensive agent ryodipine (phoridone) is superior to the familiar fluorescent probe MBA in a number of parameters relating to staining of lymphocytes. These data encourage the hope that ryodipine will succeed in replacing the MBA probe as a means of identifying T and B lymphocytes in the diagnosis both of bronchial asthma and of malignant blood diseases. Such a change would make these methods more reliable.

EXPERIMENTAL METHOD

Lymphocytes were isolated from the thymus and Peyer's patches of noninbred albino rats by the standard method [10]. Cell suspensions were prepared in Hanks' solution, pH 7.4 (Institute of Poliomyelitis and Virus Encephalitis, Moscow) and the cell concentration was determined in a Goryaev's counting chamber. To stain the cells with the fluorescent probe MBA (synthesized in B. M. Krasovitskii's Laboratory, "Monokristall" Research and Production Combine, Khar'kov) and with ryodipine (synthesized by V. V. Kastron at the Institute of Organic

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