11. P. Sai and C. Maurel, J. Immunol. Methods., 64, 189 (1983).
12. S. Srinkanta, O. P. Ganda, G. S. Eisenbarth, et al., New Engl. J. Med., 308, 322 (1983).
13. WHO Expert Committee on Diabetes Mellitus. Second Report. Technical Report Series No. 646, Geneva (1980), pp. 8-12.

PREPARATION AND STUDY OF MONOCLONAL ANTIBODIES TO SOME GANGLIOSIDES

B. B. Fuks, N. F. Avrova, L. V. Van'ko, N. A. Starosvetskaya, M. N. Boltovskaya, N. D. Durmanov, L. N. Soldatova, E. I. Zaraiskii, O. B. Karpova, N. N. Nalivaeva, and I. A. Nikolaeva

UDC 615.373:616-008.939.52-078/.012

KEY WORDS: hybridomas; monoclonal antibodies; gangliosides

The role of gangiosides in tissue interaction, in differentiation of immunocompetent cells, and in interaction of these cells with tumor cells is increasingly engaging the attention of research workers. The particular interest in gangliosides is also due to the fact that they are characteristic antigens of certain malignant neoplasms. For instance, in colorectal and certain other carcinomas, an antigen bound with the tumor is sialosyl-lacto-N-fucopentaose II, and the corresponding antigen for small-cell carcinoma of the lung is fucosyl-GM_1 [8, 9]. Immunochemical methods are nowadays widely used to study the localization and functional role of gangliosides [1, 2, 6, 7, 10]. However, the polyclonal serum antibodies avialable for use do not enable gangliosides to be accurately differentiated, and it is therefore necessary to use monoclonal antibodies for this purpose.

The aim of this investigation was to obtain monoclonal antibodies against gangliosides by immunizing animals with human melanoma cells.

EXPERIMENTAL METHOD

BALB/c mice were immunized with cultured human melanoma cells of the Mewo strain by the following scheme: first immunization — subcutaneous injection of 2×10^6 melanoma cells in Freund's complete adjuvant (1:1), followed by intraperitoneal injections of increasing numbers of cells (10^7, 2×10^7, 3×10^7) at intervals of 4 weeks. The mice were decapitated 3 days after the fourth (last) immunization, the spleen removed, and a suspension of splenocytes prepared for hybridization. The splenocytes were fused with cells of a syngeneic non-secreting P3-X63 Ag 8 myeloma in the ratio of 7:1 with the aid of polyethylene glycol (PEG-4000, from Merck, West German) by the usual method.

Antibodies secreted by the hybridomas were tested by the direct sandwich ELISA method. Individual gangliosides, isolated from the brain of the rays *Raja clavata* and *Dasyatis pastinaca*, were used. The gangliosides were extracted by the method described previously [4], then subjected to alkaline hydrolysis and purified on columns with Sephadex G-25, using chloroform—methanol—water (60:30:4.5) as the solvent. Gangliosides GM_1, GM_2, GM_3, GD_{1a}, GD_2, GD_3, GT_1, GQ_{1c}, were isolated by preparative thin-layer chromatography on silica-gel KSK (chloroform—methanol—2.5 N NH_4OH or chloroform—methanol—water, in the ratio 60:35:9, was used as the solvent). The fractions were refractionated on plates to obtain purified (95-97%) individual gangliosides. A 96-well flat-bottomed polystyrene plate (from Flow Laboratories, England) was sensitized with a 2 mM solution of the gangliosides in 96% ethanol. A panel of the above-mentioned gangliosides was used. After removal of the ethanol by evaporation, a 1% solution of bovine serum albumin (BSA) in buffered physiological saline (BPS) was added to the wells, which were then washed twice with BPS containing 0.05% Tween-20 and 2 mg/liter of BSA (BPS-T). The supernantants of the hybridomas were introduced into the wells for 1 h at 37°C, after which the plate was washed four times with cold BPS-T. Rabbit antibodies against mouse immunoglo-

Laboratory of Cellular Immunopathology and Biotechnology, Institute of Human Morphology, Academy of Medical Sciences of the USSR, Moscow. Labarotary of Neurochemistry, I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry, Academy of Sciences of the USSR, Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 1, pp. 66-68, January, 1986. Original article submitted February 26, 1985.

0007-4888/86/001-0083$12.50

TABLE 1. Reaction of Monoclonal Antibodies Produced by Hybridomas B, C, D, and Q with Gangliosides

Monoclonal antibodies	Gangliosides									
	GM_1	GM_2	GM_3	GM_4	GD_{1a}	GD_{1b}	GD_2	GD_3	GT	GQ_{1c}
B	+++	+	+++	+	—	—	—	—	±	±
C	+	+	+++	±	—	—	—	—	—	—
D	++	+	+	±	+++	+	—	+	++	+
Q	—	—	—	—	—	—	—	—	—	+++

bulins, conjugated with peroxidase, and diluted 1:1000 in BPS-T, were introduced into the wells for 40 min at 37°C, after which they were thoroughly (six or seven times) washed with cold BPS-T. Conjugation of the antibodies with horseradish peroxidase was carried out by the periodate method. Conjugates of rabbit antibodies against mouse immunoglobulins and goat antibodies against rabbit immunoglobulins were prepared. The color reaction, after being stopped with 50% H_2SO_4, was read on the "Multiscan" instrument at a wavelength of 492 nm.

The class of monoclonal antibodies was determined by solid-phase immunoassay, using rabbit antibodies against mouse immunoglobulins.

EXPERIMENTAL RESULTS

Primary screening of colonies of hybrid cells led to the discovery of 16 positive wells, which were then cloned by the limiting dilutions method. By subsequent testing and recloning, four hybridoma clones were selected which gave the strongest reaction on testing with preparations of the gangliosides. These clones, designated B, C, D, and Q, were propagated in culture and inoculated into syngeneic mice to obtain ascites fluid.

Determination of the classes of monoclonal antibodies showed that the C hybridoma synthesizes antibodies of the IgM class, whereas the B, D, and Q hybridomas synthesize antibodies of the IgG_1 class.

The results of testing the monoclonal antibodies with a set of individual gangliosides are given in Table 1. Hybridomas B and C synthesized antibodies which bound with different monosialogangliosides. Monoclonal C antibodies reacted most actively of all with gangliosides GM_3, but also gave a reaction with two other monosialogangliosides which were tested (GM_1 and GM_2). A common feature in the structure of these gangliosides is the carbohydrate sequence glucose—galactose—NeuAc. In GM_3 it is terminal, but in the other two gangliosides it lies inside the carbohydrate chain.

Antibodies produced by the hybridoma D reacted with a broad spectrum of gangliosides, but most strongly with disialoganglioside GD_{1a}. All these gangliosides possess the NeuAc—galactose sequence, but only ganglioside GD_{12} has two such sequences.

Monoclonal Q antibodies selectively bound with ganglioside GQ_{1c}.

It was shown previously that on immunization of animals even with pure preparations of individual gangliosides, antibodies in the serum were poly-specific: Besides antibodies against terminal monosaccharide sequences, antibodies also were formed against internal carbohydrate sequences [8]. The relative quantity of the different antibodies may vary considerably in sera obtained by injection of the same substance into different individuals, or even into the same animal, but at different times. As a result, the use of polyclonal serum antibodies may often give contradictory and poorly reproducible results. Monoclonal antibodies provide basically new opportunities for immunochemical analysis of gangliosides. However, the creation of a broad spectrum of monoclonal antibodies against gangliosides is a technically difficult problem which has not yet been solved.

Production of monoclonal antibodies of given specificity largely depends on the method of immunization. In the present investigation mice were immunized with melanoma cells, whose specific surface antigen is known to be ganglioside GD_3. However, none of the hybridomas which were obtained produced antibodies reacting specifically with GD_3. Only hybridoma D secreted antibodies capable of binding with gangliosides GD_3, but exhibiting their greatest activity

in the reaction with GD_{1a}. Consequently, a disadvantage of the method of immunization with whole cells, which is most frequently used to obtain monoclonal antibodies against gangliosides, is the high probability of obtaining antibodies cross-reacting with several gangliosides. This is connected with the fact that different gangliosides on the membrane surface expose the same antigenic determinants. Another method of immunization, with individual gangliosides *in vitro*, which is now being developed, may be more effective.

The hybridomas obtained in the present investigation are the first step toward the creation of a panel of monoclonal antibodies reacting with gangliosides that are widely distributed in the tissues of the body. For instance, hybridomas B and C secrete antibodies against the principal monosialogangliosides, whereas hybridomas D and Q secrete antibodies against oligosialogangliosides from various vertebrate tissues and organs.

Antibodies secreted by hybridoma Q bound only with ganglioside GQ_{1c}. The possibility of obtaining monoclonal antibodies against GQ was demonstrated previously in experiments in which mice were immunized with chick embryonic retinal cells [5]. A different structural isomer of the tetrasialogangliosides, namely GQ_{1b}, is known to predominate in adult mammalian nerve tissue. However, in the early stages of ontogeny of the higher vertebrates, tetrasialogangliosides in nerve tissue are represented mainly by ganglioside GQ_{1c}. It also predominates in the brain tissue of lower vertebrates, such as fishes. To judge from the results now obtained, GQ_{1c} is evidently expressed on the surface of melanoma cells. Further research in this direction and the use of monoclonal antibodies against gangliosides offer new prospect for the diagnosis and treatment of some malignant neoplasms [3].

LITERATURE CITED

1. B. E. Atkinson, C. S. Ernst, et al., Cancer Res., 42, 4820 (1982).
2. C. C. Curtain, Immunology, 36, 805 (1979).
3. W. Dippold, A. Knuth, and K.-H. Meyer, Cancer Res., 44, 2 (1984).
4. J. Folch-Pi, M. Lees, and G. H. Sloane-Stanley, J. Biol. Chem., 226, 497 (1957).
5. N. Kasai and R. K. Ju, Brain Res., 277, 155 (1983).
6. J. G. Kenimer, W. H. Habig, and M. C. Hardergree, Infect. Immun., 42, 942 (1983).
7. B. A. Macher, M. F. Lee, and M. A. Westrich, Mol. Cell. Biochem., 47, 81 (1982).
8. J. L. Magnani, B. Nelson, et al., J. Biol. Chem., 253, No. 23 (1982).
9. C. S. Pukel, K. O. Lloyd, et al., J. Exp. Med., 155, 1133 (1982).
10. R. Standring and A. F. Williams, Biochim. Biophys. Acta, 508, 85 (1978).