References and Notes

1. P. L. Gerfo and G. Pennington, in *Progress in Clinical Cancer*, I. M. Ariel, Ed. (Grune & Straton, New York, 1975).
2. W. D. Terry, P. A. Henkart, J. E. Coligan, C. W. Todd, *Transplant. Rev.* **20**, 100 (1974).
3. M. Herlyn, Z. Steplewski, D. Herlyn, H. Koprowski, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1438 (1979).
4. H. Koprowski, Z. Steplewski, K. Mitchell, M. Herlyn, D. Herlyn, J. P. Fuhrer, *Somat. Cell Genet.* **5**, 957 (1979).
5. A. Leibovitz, J. C. Stinson, W. B. McCombs III, C. E. McCoy, N. D. Mabry, *Cancer Res.* **36**, 4562 (1976).
6. K. Mitchell, unpublished data.
7. J. L. Magnani, M. Brockhaus, D. F. Smith, V. Ginsburg, M. Blaszczyk, K. F. Mitchell, Z. Steplewski, H. Koprowski, *Science* **212**, 55 (1981).

8. Supported in part by research grants CA-10815 and CA-21124 from the National Cancer Institute, grant RR-05540 from the Division of Research Resources, and funds from the W. W. Smith Foundation.

12 November 1980; revised 21 January 1981

A Monosialoganglioside Is a Monoclonal Antibody-Defined Antigen of Colon Carcinoma

Abstract. *The antigen of a monoclonal antibody that is specific for cells of human carcinoma of the colon is a monosialoganglioside as determined by the direct binding of antibody to thin-layer chromatograms of total lipid extracts of tissues. Binding of antibody to chromatograms is detected by autoradiography after the application of iodine-125–labeled F(ab')$_2$ of rabbit immunoglobulin G antibodies to mouse immunoglobulins.*

In (*1*) a monoclonal antibody was described that is specific for colon carcinoma cells (*2*). The binding of this antibody to antigen is inhibited by the serum of most patients with advanced colorectal carcinoma but not by the serum of normal individuals, patients with inflammatory bowel diseases, or most patients with other malignancies. The data that we present here indicate that the antigen for this antibody is a monosialoganglioside.

The binding of the hybridoma antibody to colorectal carcinoma cell line SW 1116 was unaffected by treatment of the cells with ficin, but was abolished by treatment of the cells with neuraminidase. Since this behavior suggests that the antigen may be ganglioside (*3*), total lipid extracts of cells, which contain gangliosides, were chromatographed and then tested for antigen by autoradiography.

For the autoradiography we used a modification of a method developed for the detection of gangliosides that bind to cholera toxin (*4*). The total lipid extracts of colon carcinoma cells and melanoma cells (*1*) were obtained according to a method devised for the quantitative extraction of gangliosides from brain tissue (*5*). The tissue culture cells (1 g, wet weight) were homogenized in 3 ml of H_2O at 4°C with a Potter-Elvehjem homogenizer. The homogenate was added to 10.8 ml of methanol, to which 5.4 ml of chloroform was then added with constant stirring. The extract was stirred at room temperature for 30 minutes and centrifuged at 15,000*g* for 10 minutes. The pellet was rehomogenized in 2 ml of H_2O and extracted as above with 8 ml of a mixture of chloroform and methanol (1:2 by volume).

The supernatant solutions from both extractions were combined and evaporated under a stream of dry nitrogen, and the residue was dissolved in chloroform and methanol (2:1 by volume) for application to a thin-layer chromatography sheet (*6*). Samples of 1 μl of total lipid extract suitably diluted were spotted 1.5 cm from the bottom of the sheet which was then clamped in a sandwich chamber and developed in a chromatography tank containing chloroform, methanol, and 0.25 percent KCl (60:35:8 by volume). The chromatogram was air-dried, and then soaked for 10 minutes at 4°C in 0.01*M* sodium phosphate buffer, *p*H 7.2, containing 0.15*M* NaCl, 1 percent polyvinylpyrrolidone (molecular weight 40,000, pharmaceutical grade; Sigma) and 0.1 percent sodium azide (buffer A). The wet chromatogram was laid horizontally on a slightly smaller, parafilm-covered glass plate. Serum-free hybridoma culture medium containing about 10 μg of antibody per milliliter (*1*) was diluted 1:4 with buffer A and gently pipetted onto the chromatogram (about 50 μl per square centimeter of chromatogram). After incubation in a humid atmosphere for 6 hours at 4°C, the chromatogram was washed by dipping in six successive changes of 0.01*M* sodium phosphate buffer, *p*H 7.2, containing 0.15*M* NaCl (buffer B). The chromatogram was then laid horizontally as before and immediately layered with ^{125}I-labeled F(ab')$_2$ of rabbit immunoglobulin G antibodies to mouse immunoglobulins in buffer A (10^6 counts per minute per milliliter; about 50 μl/cm^2 of chromatogram). After incubation in a humid atmosphere for 12 hours at 4°C, the chromatogram was washed six times in buffer B, air-dried, and exposed to XR-2 x-ray film (Eastman Kodak) for 50 hours.

As shown in Fig. 1, antigen is detected in extracts of colorectal carcinoma cells (lanes 4 to 6) but not in extracts of human melanoma cells (lanes 1 to 3), which do not bind antibody (*1*). The antigen migrates between the standard gangliosides G_{M1} and G_{D1a} under the conditions described. Binding of antibody to the carcinoma cell extract is abolished by treatment of the extract with neuraminidase.

When the total lipid extract was chro-

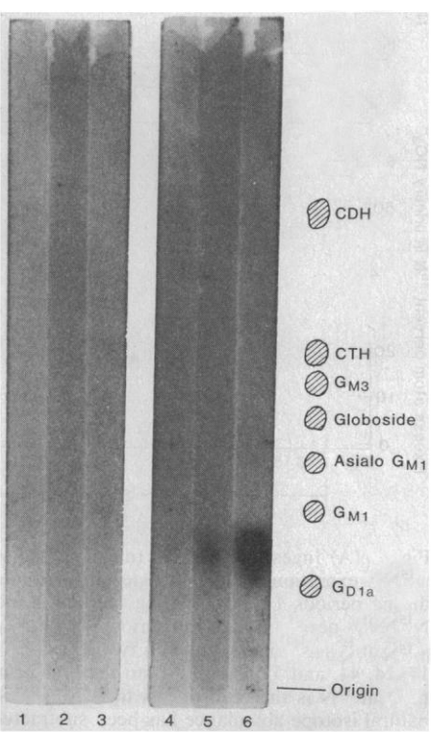

Fig. 1. Autoradiography of the antigen detected by a monoclonal antibody to colorectal carcinoma as described in the text. The amounts of extract chromatographed, expressed as the volume of packed cells from which the extract was obtained, are as follows: lanes 1, 2, and 3 contain lipids from 0.02, 0.1, and 0.5 μl of packed melanoma cells, respectively; lanes 4, 5, and 6 contain lipids from 0.02, 0.1, and 0.5 μl of packed colorectal carcinoma cells, respectively. The positions of some standard gangliosides and neutral glycolipids after chromatography are shown on the right. Abbreviations are: *CDH*, Galβ1-4Glcβ1-1Ceramide; *CTH*, Galβ1-4Galβ1-4Glcβ1-1Ceramide; G_{M3}, NeuNAcαa2-3Galβ1-4Glcβ1-1Ceramide; *Globoside*, GalNAcβ1-3Galα1 - 4Galβ1 - 4Glcβ1 - 1Ceramide; *Asialo* G_{M1}, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Ceramide; G_{M1}, Galβ1-3GalNAcβ1-4[NeuNAcα2-3]Galβ1-4Glcβ1-1Ceramide; and G_{D1a}, NeuNAcα2 - 3Galβ1-3GalNAcβ1 - 4[NeuNAcα2 - 3]Galβ1-4Glcβ1-1Ceramide.

0036-8075/81/0403-0055$00.50/0

matographed on DEAE-Sephadex (*7*), the antigen became bound to the DEAE-Sephadex and was eluted from the resin in the monosialoganglioside fraction by 0.02*M* ammonium acetate in methanol. No antigen was detected either in the disialoganglioside fraction eluted by 0.12*M* ammonium acetate in methanol or in the tri- and tetrasialoganglioside fraction eluted by 0.5*M* ammonium acetate in methanol. On the basis of this property and its chromatographic mobility (Fig. 1), we conclude that the antigen is probably a large monosialoganglioside.

The antigen is not detected by the autoradiographic method described here in ganglioside mixtures from human tissues other than colorectal carcinoma (*8*). However, the antigen is present in human meconium, which is a rich source of fetal glycolipids (*9*). The glycolipids of tumor cells differ from their normal counterparts (*10*) and some may reflect their embryonic origin.

JOHN L. MAGNANI
MANFRED BROCKHAUS
DAVID F. SMITH
VICTOR GINSBURG
National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205
MAGDALENA BLASZCZYK
KENNETH F. MITCHELL
ZENON STEPLEWSKI
HILARY KOPROWSKI
Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

References and Notes

1. H. Koprowski, M. Herlyn, Z. Steplewski, H. F. Sears, *Science* **212**, 53 (1981).
2. A. Leibovitz, J. C. Stinson, W. B. McCombs III, C. E. McCoy, N. D. Mabry, *Cancer Res.* **36**, 4562 (1976).
3. C.-M. Tsai, D. A. Zopf, R. K. Yu, R. Wistar, Jr., V. Ginsburg, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4591 (1977).
4. J. L. Magnani, D. F. Smith, V. Ginsburg, *Anal. Biochem.*, in press.
5. L. Svennerholm and P. Fredman, *Biochim. Biophys. Acta* **617**, 97 (1980).
6. Eastman Kodak Co., chromatogram 100 μm thick, 10 by 10 cm.
7. R. W. Ledeen and K. Yu, "Methods of isolation and analysis of gangliosides," *Research Methods in Neurochemistry*, N. Marks and R. Rodnight, Eds. (Plenum, New York, 1978), vol. 4, pp. 371–410.
8. The human erythrocyte gangliosides were kindly provided by D. Marcus and S. Kundu, Baylor College of Medicine. The human brain gangliosides were kindly provided by R. K. Yu, Yale University School of Medicine. Human meconium was obtained through the courtesy of C. Hultzen, Sibley Memorial Hospital, Washington, D.C.
9. K.-A. Karlsson and G. Larson, *FEBS Lett.* **87**, 283 (1978).
10. S.-I. Hakomori, *Biochim. Biophys. Acta* **417**, 55 (1975).
11. Supported in part by research grants CA-10815 and CA-21124 from the National Cancer Institute, grant RR-05540 from the Division of Research Resources, and funds from the W. W. Smith Foundation.

12 November 1980

Nitrate Synthesis in the Germfree and Conventional Rat

Abstract. *Metabolic balance studies show that germfree and conventional Sprague-Dawley rats synthesize nitrate. Equivalent results for germfree and conventional rats eliminate the microflora as obligatory components of nitrate production. Nitrate synthesis appears to be a mammalian process.*

Nitrate (NO_3^-) and nitrite (NO_2^-) (*1*) react in the body to form carcinogenic nitrosamines (*2*). The diet is not the only source of NO_3^- for such reactions, as humans and laboratory animals on low-NO_3^- diets can excrete more NO_3^- than they ingest (*3*, *4*). We report here isotopic studies with rats that confirm that NO_3^- is synthesized in the body and show by the use of germfree rats that the flora is not obligatory for its formation.

Germfree or conventional (cesarean-derived, specific pathogen–free) male Sprague-Dawley rats (initially weighing 150 g; Charles River Breeding Laboratories, Wilmington, Massachusetts) were housed individually in metal metabolism cages and were given unlimited amounts of distilled water and a casein- and cornstarch-based, low-NO_3^-, low-NO_2^- diet (*5*). Germfree rats were maintained in flexible film isolators (*6*). The basal diet was supplemented as indicated with either 0.001 or 0.005 percent $Na^{15}NO_3$ (KOR Isotopes, Cambridge, Massachusetts). Food consumption was determined at 24-hour intervals for the conventional animals and estimated to be comparable for germfree animals (*7*). Urine and feces were collected during each 24-hour period. Urine for the conventional animals was preserved with isopropyl alcohol (final concentration, ≥ 8 percent). All samples were maintained at −15°C until analyzed.

We determined the NO_3^- content of food, urine, or feces by reducing it to NO_2^- on a cadmium column and assaying the NO_2^- by diazotization and coupling with a Griess reagent (*8*). The relative abundance of $^{15}NO_3^-$ and $^{14}NO_3^-$ was determined by conversion of NO_3^- to nitrobenzene (*9*), which was then analyzed by gas chromatography–mass spectrometry (GC-MS) (*10*). This method was also used to confirm the reliabil-

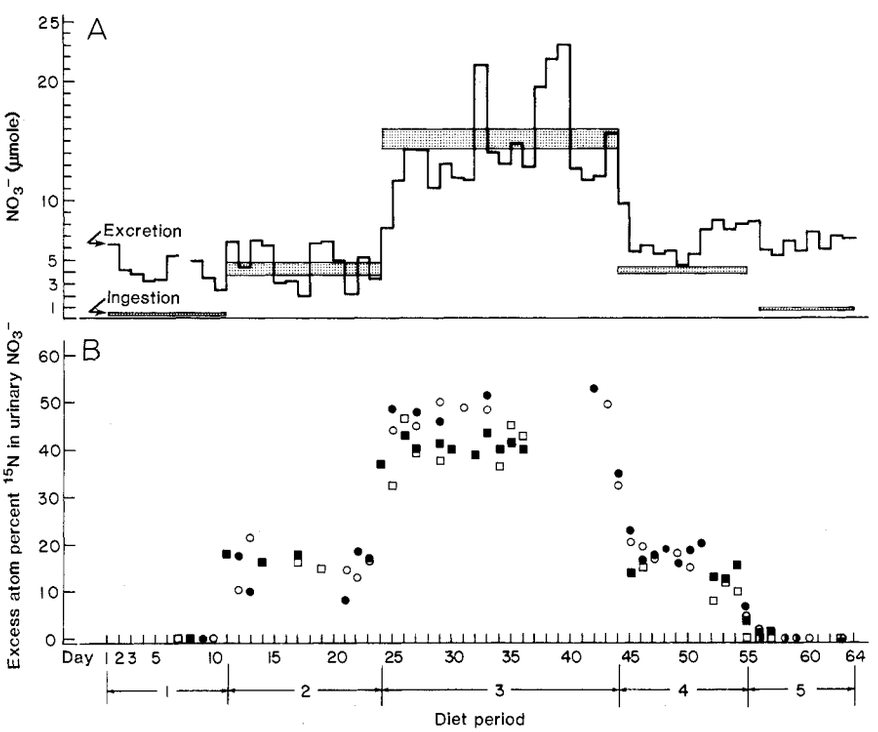

Fig. 1. (A) Ingestion of NO_3^- (mean ± the standard deviation) (the five shaded blocks) and urinary excretion of NO_3^- for one germfree rat. The minimal NO_3^- diet (Table 1) was consumed during periods 1 and 5; during periods 2 and 4, this diet was supplemented with 10 μg of $Na^{15}NO_3$ per gram and, for period 3, with 50 μg of $Na^{15}NO_3$ per gram. (B) Daily excess atom percent ^{15}N in urinary NO_3^- for two germfree (○, ●) and two conventional (□, ■) rats. Days 1, 11, 24, 44, and 55 correspond to the first 24-hour periods after diet change. The excess atom percent ^{15}N is the ratio of ^{15}N to (^{14}N + ^{15}N) in urinary NO_3^- from which this ratio for the natural isotope abundance has been subtracted.

0036-8075/81/0403-0056S00.50/0