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NEW CARBOHYDRATE ANTIGEN FOUND IN LARGE GLYCOPEPTIDES OF TERATOCARCINOMA CELLS

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SUMMARY—— From rabbit antiserum against Dolichos agglutinin receptors of murine teratocarcinoma OTT6050, an antibody preparation has been isolated by affinity chromatography on the large glycopeptides of the teratocarcinoma. The antibody recognized carbohydrate antigen(s), which was expressed on embryonal carcinoma cells and primitive endodermal cells of teratocarcinomas, 2- to 4- cell embryos, and on GRSL leukemia cells, but not on many adult cells. The antigen was different from several carbohydrate antigens such as ABH, Forssman, Ii and SSEA-1 and will be useful as a marker of differentiation and tumorigenesis in a certain system.

Teratocarcinomas are malignant tumors containing stem cells plus differentiated tissues derived from each of the three germ layers. The stem cells called embryonal carcinoma cells resemble multipotential cells of early embryos and have been conveniently used as a model system to study cell differentiation and tumorigenesis (1). Cell surface glycoproteins of embryonal carcinoma cells contain unusually large carbohydrate chains, whose core structure is composed of galactose and N-acetylglucosamine (2,3). Because of the paucity of these carbohydrates in adult tissue and of their high-molecular-weight nature, they have been expected to carry some carbohydrate antigens with developmentally regulated distribution. In this communication, we demonstrate that the large carbohydrates indeed have such antigenic determinant(s). The new carbohydrate antigen will be shown to be different from other surface antigens of embryonal carcinoma cells (4,5,6,7) and some carbohydrate antigens found in adult cells (6,8).

Abbreviations used : DBA, Dolichos biflorus agglutinin ; PBS (-), Dulbecco's phosphate buffered saline without Ca^{++} and Mg^{++} ; PBS (+), Dulbecco's phosphate buffered saline ; TC, teratocarcinoma-derived carbohydrate ; SDS, sodium dodecyl sulfate.

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Materials and Methods

Large Glycopeptides from Teratocarcinoma OTT6050. Particulate fraction was prepared from 55g of teratocarcinoma OTT6050 (9) grown in peritoneal cavity of 129/SV mice by homogenization in PBS (-) (10) followed by ultracentrifugation. The particulate fraction was dissolved in 2 % Triton X-100 in 0.01 M Tris-HCl, pH 7.6 containing 0.15 M NaCl. The supernatant collected by ultracentrifugation was mixed with 4 volumes of cold acetone. The precipitate was dried in vacuo. The acetone powder (920 mg) was suspended in 92 ml of 0.1 M Tris-HCl buffer, pH 8.4, mixed with 46 mg of crystalline papain in 4.6 ml of 0.2 M Tris-HCl buffer, pH 8.4, containing 10 mM cysteine, and was incubated at 37°C. After 24 h 46 mg of papain and after 48 h 46 mg of Pronase E were added. After further incubation for 24 h, the mixture was concentrated to 8.0 ml and applied to a column of Sephadex G-50 (1.5 x 90 cm) equilibrated with 0.05 M ammonium acetate buffer, pH 6.0. After elution with the same buffer, glycopeptides eluted in the void volume were dialyzed against distilled water and concentrated to 12 ml, and then extracted with chloroform-methanol (2 : 1, v/v). The aqueous layer was evaporated to dryness, dissolved in 2.0 ml of 0.01 M Tris-HCl buffer, pH 7.5 containing 0.1 M NaCl, and applied to a column of DEAE-Sephadex A-25 (1.5 x 5 cm) equilibrated with the same buffer. The column was washed with 3 column volume of the same buffer and the unabsorbed fraction was collected as the large glycopeptides.

Purification of the Anti-carbohydrate Antibody. Receptors for Dolichos biflorus agglutinin was prepared as described previously (11). New Zealand White rabbits were immunized with the receptors (0.2 mg protein) in complete Freund's adjuvant by injection in the foot pads. After 3 weeks, the animals received two booster injections at 2 week intervals using the same amount of the antigen. Serum was collected 10 days after the last immunization. The large glycopeptides prepared as described in the previous section were coupled with Sepharose 4B activated with CNBr at the ratio of 0.65 mg neutral sugars of the glycopeptides per 1 ml of the resin at 4°C overnight in 0.1 M sodium bicarbonate buffer, pH 8.7 containing 0.5 M NaCl. After terminating the coupling reaction with 0.1 ml of 1 M ethanolamine-HCl, pH 8.0, the agarose beads were washed with 30 ml of 0.1 M sodium bicarbonate buffer, pH 8.7 containing 0.5 M NaCl and with 50 ml of distilled water. Two ml of anti-DBA receptor serum was applied to the large glycopeptide-Sepharose column (0.9 x 1.6 cm) equilibrated with PBS (+) and the column was washed with 140 ml of PBS (+). Anti-carbohydrate antibodies were eluted with 4 ml of 3 M KSCN in PBS (+), dialyzed against 1 l of PBS (+) for 2 days changing the PBS (+) every 12 h and were concentrated to 2 ml.

Indirect Immunoprecipitation. [^{3}H]-galactose-labeled large glycopeptides were prepared as described previously (12) from F9 cells labeled with 6-[^{3}H]-galactose (1 Ci/mmole, Radiochemical Centre). The glycopeptide (10,000 cpm) in 10 µl of H_2O was mixed with 50 µl of undiluted antiserum or the anti-TC antibody solution and left for 1 h at 4°C. The immune complex was precipitated by the addition of 200 µl of 10 % suspension (v/v) of formalin-killed Staphylococcus aureus (13) in PBS (+). After 1 h at 4°C, 5 ml of PBS (+) was added and the immune complex was collected by centrifugation, and was washed two times with 5 ml of PBS (+).

Results and Discussion

Glycoproteins with terminal α-N-acetylgalactosaminyl residues were isolated from teratocarcinoma OTT6050 by affinity chromatography on agarose -conjugated Dolichos biflorus agglutinin (DBA) which is a lectin specific to α-N-acetylgalactosamine residue (14). Rabbit antiserum raised against the DBA receptors contained antibodies reacting with the large glycopeptides of

Table I Indirect Immunoprecipitation of the Large Glycopeptides from F9 Embryonal Carcinoma Cells by the Affinity-purified Anti-carbohydrate Antibodies.

Serum or antibodies	Large glycopeptides	Per cent of the [^{3}H]-galactose-labeled glycopeptides precipitated
Experiment A[1)]		
Anti-DBA receptor serum	mixture	21.6
Anti-sheep erythrocytes	"	1.5
non-immune serum	"	1.6
The affinity purified antibodies	"	25.1
Experiment B[2)]		
The affinity purified antibodies	mixture	27.5
"	mixture, periodate oxidized	1.2
"	bound to DBA-agarose	68.4
"	unbound to DBA-agarose	9.5

1) Experiments were performed as described in Materials and Methods.

2) [^{3}H]-galactose-labeled large glycopeptides (1.0×10^5 cpm) were applied to a column of DBA-agarose (0.6 x 1.5 cm) equilibrated with PBS (+). The column was washed with 5 ml of PBS (+) and then eluted with 0.8 ml of PBS (+) containing 0.1 M N-acetylgalactosamine. Of the total large glycopeptides, about 20 % was bound to and eluted from the column. Periodate oxidation was performed in 0.2 ml of reaction mixture containing 98,000 cpm of the glycopeptides, 35 mM sodium metaperiodate and 35 mM sodium acetate buffer, pH 4.5 at 4°C for 8 h. Ethylene glycol (10 µl) was added, and the reaction mixture was dialyzed against H_2O. For the immunoprecipitation experiment, 200 µl of the large glycopeptides in PBS (+) thus treated or fractionated and 50 µl of anti-TC antibody were incubated for 1 h at 4°C. Other experimental conditions were the same as described in Materials and Methods.

F9 embryonal carcinoma cells. The immunological reaction could be demonstrated by indirect immunoprecipitation employing S. aureus (Table I, Experiment A). Neither non-immune rabbit serum nor rabbit anti-sheep erythrocytes, which contains anti-Forssman antibodies, was reactive to the large glycopeptides. The specific antibodies reacting with the large glycopeptides could be isolated by immuno-affinity chromatography on immobilized large glycopeptides of teratocarcinoma OTT6050 (Table I, Experiment A).

The antigenic determinant(s) should be carbohydrate(s), since the glycopeptides were prepared by extensive pronase digestion. Furthermore, period-

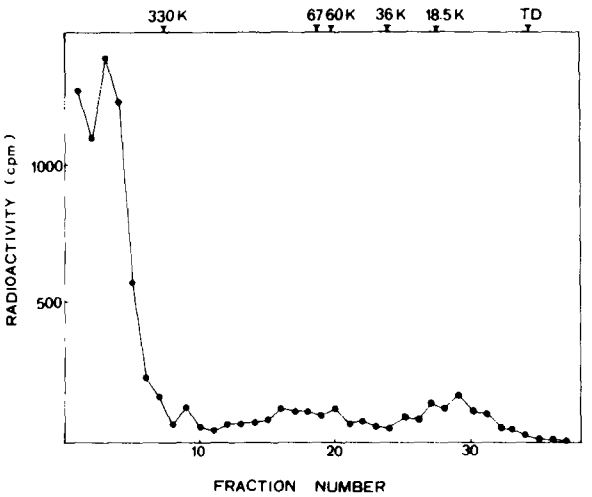

Fig. 1. SDS polyacrylamide gel electrophoresis of TC antigen isolated from galactose-labeled teratocarcinoma OTT6050 cells. The teratocarcinoma cells were cultured in vitro, labeled with [^{3}H]-galactose as described previously (11), and were extracted with 2 % Triton X-100 in 0.01 M Tris-HCl buffer, pH 7.6 containing 0.15 M NaCl. TC antigen was isolated by indirect immunoprecipitation as described in Materials and Methods except that 50 µl of the Triton extract (6.3 x 10^5 cpm) was used, that the buffer used to suspend S. aureus was 0.01 M Tris-HCl, pH 7.6 containing 2 % Triton X-100 and 0.15 M NaCl and that the precipitates were washed at first with 0.01 M Tris-HCl, pH 7.6 containing 2 % Triton X-100 and 0.15 M NaCl, then with the buffer containing 0.1 % Triton X-100 and 0.15 M NaCl and finally with PBS (+). [^{3}H]-galactose-label recovered in the immunoprecipitate was 3.4 % of the radioactivity in the Triton extract. The control value obtained using non-immune serum was 0.4 %. The immunoprecipitate (1.1 x 10^4 cpm) was analyzed by SDS gel electrophoresis on 3.5 % gel (0.5 x 7.5 cm) according to Fairbanks et al. (19). Gels were cut into 2 mm slices, extracted with 0.6 ml of 0.01 M Tris-HCl buffer pH 7.6 containing 1 % SDS and 0.15 M NaCl for 5 days and counted. Standard substances used were thyroglobulin (molecular weight 330,000) bovine serum albumin (67,000), catalase (60,000), lactate dehydrogenase (36,000) and ferritin (18,500). The location where the standard substances migrated was shown in the figure. TD: Tracking dye (Pyronin G, Merck).

ate oxidation abolished the antigenic activity (Table I, Experiment B). We tentatively named the carbohydrate antigen(s) as TC (teratocarcinoma-derived carbohydrate) antigen. When the large glycopeptides were fractionated into those binding to DBA agarose and those unbinding to the resin, TC antigen was found to be preferentially expressed in the former fraction (Table I, Experiment B). The result may suggest that the antigenic determinant(s) involve(s) the DBA binding site, namely N-acetylgalactosamine. Intact molecules carrying TC antigen were isolated by solubilization in Triton X-100 followed by indirect immunoprecipitation. The antigens from teratocarcinoma OTT6050

Table II Distribution of TC Antigen.

Positive sites	Murine cells
	F9 embryonal carcinoma cells[1]; embryonal carcinoma and endodermal cells of teratocarcinoma OTT6050[1]; GRSL leukemia cells[2]; 2-4 cell embryos[3].
Negative sites	Murine cells
	Lymph node cells[2]; spleen cells[2]; thymocytes[2]; erythrocytes[2,4]; colon[5]; uterus[5]; testis[5]; morulae[3].
	Cells from other sources
	Sheep erythrocytes[2,4]; human erythrocytes of A, B, AB and H specificities[2,4].

Distribution of TC antigen was studied by indirect immunofluorescence. In case of erythrocytes, hemagglutination test was also performed. Cells and tissues of the mouse were taken from 129/SV mice except for the embryos.

1) F9 cells and teratocarcinoma OTT6050 grown as described before (11) on glass cover-slips were incubated with 50 µl of the anti-TC antibodies or normal rabbit serum both diluted 20- fold in PBS (+) for 1 h at 4°C. After washing the cell layers by PBS (+), they were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Miles) diluted 10- fold for 1 h at 4°C. After washing twice with PBS (+), cells were examined by a fluorescence microscope with epi-illumination.

2) Fifty µl of 1 % cell suspension (v/v) was mixed with 50 µl of the anti-TC antibodies or normal rabbit serum both diluted 10- fold. Other procedures were similar to those described above.

3) Preimplantation embryos were flushed from the oviduct of ICR mice. After zonae pellucidae were removed by a brief treatment with 0.5 % pronase (20), the embryos were incubated for 2 h at 37°C in Whitten's medium (21). The staining procedure was essentially the same as in 2).

4) Fifty µl of 1 % (v/v) suspension of erythrocytes in PBS (+) was mixed with 50 µl of anti-TC antibody diluted 4- fold with PBS (+) and was allowed to stand for 1 h at 4°C. Hemagglutination was examined microscopically.

5) Cryostat sections were stained by indirect immunofluorescence. Detailed procedures will be described elsewhere.

(Fig. 1) and from F9 (data not shown) both migrated as glycoproteins of apparent molecular weight more than 300,000 upon SDS gel electrophoresis.

Distribution of TC antigen was studied by indirect immunofluorescence (Table II). The antigen was detectable in both the stem and endodermal cells of teratocarcinoma OTT6050. It was detectable in 2-4 cell embryos, but disappeared from morulae. Absence from sheep and human erythrocytes distin-

guished the antigen from Forssman, ABH and I antigens. The antigen was different from i antigen, since the latter antigen is not detectable in embryonal carcinoma cells (6). Cells from several organs of adult mice were also negative in the antigen. On tissue sections, the antigen was detectable in severely restricted regions expressing DBA receptors such as renal collecting tubules (data not shown). Full description on the antigenic distribution in embryonic and adult tissues will appear elsewhere. The antigen was expressed on GRSL leukemia cells, which also express DBA receptors (15). Furthermore, anti-TC antibodies with the capabilities of the staining of teratocarcinoma OTT6050 were completely absorbed by GRSL cells. These results again suggest that TC antigen might be directed against binding sites of DBA, namely N-acetylgalactosamine. However, it is worth noting that DBA reacts with Forssman and A antigens, while the anti-TC antibody does not. Therefore, the anti-TC antibody is more strict in specificity as compared to DBA even if the antigenic sites and the receptor sites are identical in certain cells such as teratocarcinomas.

We have here reported an antigen (or antigens) in the large carbohydrate chains of teratocarcinoma cells. The antigen is different from other embryonic antigens known to occur in embryonal carcinoma cells, namely SSEA-1 (5) and F9 antigen (4). The latter two antigens are detected on morulae (4,5,16), but TC antigen was not. Furthermore, molecular weight of intact F9 antigen is only 40,000 (17) and SSEA-1 has been reported to be glycolipids (5). The antigenic determinant of SSEA-1 is Fucα1$\rightarrow$3GlcNAc (18), while that of TC antigen might involve N-acetylgalactosamine. This newly described antigenic determinant preferentially expressed in certain embryonic and malignant cells will be helpful in a number of embryological and oncological studies. Furthermore, the present results illustrate the utility of the large glycopeptides of teratocarcinoma cells as the ligand for the affinity chromatography of anti-carbohydrate antibodies and the reagent to detect such antibodies.

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