Tk, a New Colon Tumor-associated Antigen Resulting from Altered O-Glycosylation

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INTRODUCTION

Aberrant glycosylation is a well-known characteristic of cancer cells, and many tumor-associated antigens defined by mAbs raised against cancer cells are cell surface carbohydrate epitopes (1, 2). Regarding digestive cancers, most epitopes recognized are related to blood group antigens. Indeed, overexpression of ABH, Lewis b, and Lewis y antigens occurs in distal colorectal tumors. Similarly, expression of sialylated Lewis b and Lewis x is strongly increased (3–11). Lewis y antigens occurs in distal colonic tumors. Similarly, expression of sialyl Lewis a and sialyl Lewis X is strongly increased (3–11).

ABSTRACT

Erythrocyte polyagglutination antigens T and Tn are truncated O-glycan chains that are also carcinoma-associated antigens. We investigated whether Tk polyagglutination antigen could similarly be a carcinoma-associated marker and a target of immunotherapy. Monoclonal antibody LM389 was raised against Tk erythrocytes and tested by immunohistochemistry. LM389 strongly reacted with 48% human colorectal carcinomas. Labeling of normal tissues was visible on epithelial cells, mainly digestive, but was confined at a supranuclear level. Expression of the antigen on cloned human carcinoma cells correlated with sialosyl-Tn expression. O-Sialo-y-glycoprotein endopeptidase treatment revealed that on carcinomas and cell lines, the epitope was present on O-glycans. Antibody specificity was determined using synthetic carbohydrates. Direct binding and inhibition studies indicated that LM389 best ligands were terminated by two branched N-acetylgalactosamine units. Screening of murine cellular cell lines with LM389 allowed development of an experimental model with Tk-positive and -negative cells in syngeneic BDIX rats. Vaccination of rats with Tk erythrocytes provided a protection against growth of rat Tk-positive, but not of Tk-negative, tumor cells in association with the development of antibodies. Taken together, the results indicate that Tk polyagglutination antigen is a new colorectal carcinoma-associated antigen, absent from the normal cell surface, resulting from alteration of O-glycans biosynthesis and with potential as a target of immunotherapy.
This mAb was identified as a murine IgM x using a dipstick isotyping kit (Life Technologies, Inc., Paisley, Scotland) and in tests with in vivo modified T, Tk, Th, Tn, Cad, Sd, and acquired B cells, the mAb reacted only with the Tk cells. Additionally, using red cells modified in vitro by B. fragilis supernatant, endo-β-galactosidase, and neuraminidase, only the first two of these, the Tk-activated cells, were agglutinated (24).

Tissue Samples. Normal tissues were obtained either from surgical specimens or from kidney donors 5 min after death. These last human samples were obtained before the law 88-1138 (December 20, 1988) concerning resection of organs or from kidney donors 5 min after death. These last human samples were obtained before the law 88-1138 (December 20, 1988) concerning resection of organs or from kidney donors 5 min after death.

Immunohistochemistry. Sections (5 μm) were rehydrated in graded ethanol and washed in PBS. They were then incubated in methanol/H2O2, 0.3% for 20 min to block endogenous peroxidase, and washed 5 min in PBS. The tumor or normal tissue sections were then covered with PBS:3% BSA for 20 min at room temperature in a humidified atmosphere. After washing in PBS, sections were incubated with the primary antibody diluted in PBS:1% BSA at 4°C overnight. Sections were then rinsed twice with PBS and incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 1 h at room temperature. After washing in PBS, sections were covered with peroxidase-conjugated avidin (Vector) for 45 min and washed with PBS, and reactions were revealed with 3-amino-9-ethylcarbazol. Counterstaining was performed with 1% Harris hematoxylin. To release O-glycans immediately after rehydration, sections were incubated in the presence of O-sgp (Cedarlane, Hornley, Ontario, Canada) and diluted in RPMI 1640 at a concentration of 120 μg/ml for 4 h at 37°C. Control sections were incubated similarly in the absence of the enzyme. Sections were then washed three times in PBS, incubated in PBS:3% BSA, and stained with the anti-Tk as described above. Within each tumor section, the percentage of positive cells was semiquantitatively estimated by two independent observers at low power field (×10). Tumors were scored as negative below 5% positive cells, as weakly positive below 25% positive cells, as moderately positive below 75% positive cells, and as strongly positive from 75 to 100% positive cells.

Cell Culture and Selection of Tk-positive Cells. The SW707 human colon carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). LSC and LSB are two clones derived from the human colon adenocarcinoma cell line LS174T. These two clones have been selected for their positive and negative expression of Tn and STn antigens, respectively (25). These cells were kindly provided by Dr. S. H. Itzkowitz (Department of Pathology, Memorial Sloan-Kettering, San Jose, CA).

Cell sorter FACScan (Becton Dickinson, San Jose, CA). Medium was RPMI 1640. Media were supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. (Life Technologies, Inc., Cergy-Pontoise, France). They were subcultured at confluency (3 days) by a 1-h incubation at 37°C, followed by an overnight incubation at 4°C. After three washes with the same buffer, a 30-min incubation with the secondary anti-mouse FITC-labeled antibody (Sigma) was performed. After washings, fluorescence analysis was performed on a fluorescence-activated cell sorter FACScan (Becton Dickinson, San Jose, CA).

Western Blot Analysis. For Western blotting, total proteins were solubilized as cells reached confluency by a 30-min incubation at 4°C in PBS (pH 7.4) containing 1 mmol/L EDTA, 10 mmol/L NaF, and 0.1% sulfobetaine-14. The preparations were centrifuged at 13,000 × g for 15 min. Protein concentration of supernatants was measured using bicinchoninic acid. Twenty-five μg of proteins were subjected to electrophoresis in 5–15% SDS-polyacrylamide gels in the presence of 5% β-mercaptoethanol. Separated proteins were electrotherophoretically transferred to nitrocellulose filters in 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol at 200 mA for 1 h. The efficiency of transfer was monitored by Ponceau S staining of the nitrocellulose filter. After transfer, membrane strips were incubated with O-sgp at a concentration of 120 μg/ml in PBS:1% Triton X-100 for 6 h at 37°C. A control was done by incubating the membrane in the same conditions without the enzyme. Afterward, blots were incubated for 1 h in 3% defatted milk in PBS. The anti-Tk and anti-actin (Boehringer Mannheim, Mannheim, Germany) antibodies were then incubated (v/v) overnight at 4°C in PBS containing 1% defatted milk. After three washes in PBS/Tween 0.05%, a 2-h incubation was performed with an antirabbit peroxidase-labeled antibody diluted in PBS containing 3% BSA. After washing, the bound antibodies were revealed by chemiluminescence using the ECL kit from Amersham (Little Chalfont, United Kingdom).

Inhibitions of Glycosylation. To inhibit the maturation of N-glycans, cells were cultured for 48 h in the presence of DMJ from Boehringer Mannheim and cultured in culture medium at a concentration of 1 mmol/L O-Glycans from the cell surface were also cleaved by the O-sgp enzyme (Cedarlane). For this, confluent cells in six-well plates were incubated for 4 h with the enzyme, diluted in fetal calf serum-free medium at a concentration of 120 μg/ml. Cells were then tested by flow cytometry with the anti-Tk mAb or with the lectin L-PHA as a control for efficiency of the DMJ treatment.

Determination of mAb LM389 Specificity. For adsorption on SYN-SORB, synthetic oligosaccharides coupled to a silica solid support (SYN-SORB) were obtained from Chembio Ltd. and from Dr. R. U. Lemieux (Edmonton, Alberta, Canada). One hundred μg of mAb LM389 as a cell supernatant diluted 1:2 in PBS containing 0.1% gelatin were incubated on 10 μg of wet SYN-SORB and incubated for 1 h at room temperature under gentle agitation. After centrifugation for 3 min at 3000 × g, the supernatant was recovered and tested by flow cytometry on PROb cells. Binding of the antibody was quantified by the decrease in mean fluorescence intensity relative to that given by the supernatant incubated on the same solid support lacking a coupled oligosaccharide. The following immobilized oligosaccharides were used: Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ-R, Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ-R, and GlcNAcβ1–3Galβ1–4Glcβ-R. For these, the linking arm and the silica solid support. Binding to Polyalcaramide-based Neoglyconjugates. Neoglycoconjugate probes were prepared by conjugating synthetic oligosaccharides to PAA. Three types of spacers between the oligosaccharides and the polyaclaramide were used: Sp3, -O(CH2)3; Sp2, -O(CH2)2; and Sp1, -O(CH2)2(CHOH)2CHNH2CH2. The methods for synthesis of oligosaccharides, of poly(4-nitrophenylacrylate), and for preparation of oligosaccharide polyaclaramide conjugates have been reported (27). All of the probes contained equal amounts of oligosaccharide (20 mol%). The neoglycoconjugates used in the present study are listed in Table 4. Reactivity of mAb LM389 toward these probes was tested by ELISA. Probes were coated onto 96-well plates (Nunc, Roskilde, Denmark) at 10 μg/ml in sodium carbonate buffer 0.05 mol/l (pH 9.6) by a 1-h incubation at 37°C, followed by an overnight incubation at 4°C. Plates were then incubated for 1 h at 37°C in PBS containing 1% BSA.
Between each step, plates were washed three times with PBS:0.1% Tween 20. mAb LM389, as a culture supernatant, was added in 2-fold serial dilutions from 1:10 in PBS:1% BSA and incubated for 1 h at 37°C. Its binding was then revealed by addition of peroxidase-labeled antimouse (Vector) diluted to 1:3000 in PBS:1% BSA for 1 h at 37°C. After washings, plates were incubated with orthophenylenediamine (Sigma) at 0.4 mg/ml in sodium citrate buffer, 10 mMol/l, pH 6.0. Reactions were stopped by addition of 50 μl of H₂SO₄ at 30% and read at 405 nm. Inhibitions of binding were also performed by ELISA. In this case, plates were coated with the probe GlcNAcβ₁-₄GlcNAc-Sp⁻³-PAA, as described above. Then, after a blocking step, inhibitory substances, either as PAA neoglycoconjugates or as monomeric oligosaccharides (listed in Table 4) were added in a volume of 50 μl in 2-fold serial dilutions starting from 1 mmol/l. Fifty μl of LM389 cell supernatant, diluted 1:5 in PBS:1% BSA, were then added in each well to obtain a 1:10 final dilution. Incubation was performed for 1 h at 37°C. Antibody binding was then revealed as described above.

**Rat Immunization Experiments.** Inbred BDIX rats were purchased from Iffa-Credo (L’Abresle, France), housed and bred under standard conditions in our laboratory. Rats, 2–3 months of age, were used. Groups of five or six rats were prepared and received i.v. penile injections or i.p. injections three times, one week apart, of 50 μl of packed human red cells diluted with PBS to a final volume of 250 μl. i.v. injections were performed under anesthesia. The erythrocytes were either untreated, treated with papain, treated with endo-β-galactosidase, or with both enzymes. Treatment with papain was performed by adding 125 μg of papain (Blood Transfusion Center, Nantes, France) to a 250-μl suspension of cells for 15 min at 37°C. For treatment with endo-β-galactosidase, 5 μl of the enzyme from *B. fragilis* (Calbiochem, La Jolla, CA) in 250 μl of carbonate buffer 0.05 mol/l (pH 5.8) were added to a 250-μl suspension of cells and incubated for 3 h at 37°C. After treatment, cells were washed three times with PBS. Two weeks after the last injection, animals received 1 × 10⁶ PROb or A15A5 cells suspended in 1 ml of RPMI 1640. Tumor cells were injected either s.c. in the flank of animals in the case of i.v. immunization, and tumors were measured every week with calipers, or i.p. in case of i.p. immunization, in which case rat survival was monitored. These experiments were performed in agreement with the rules of the French Ministry of Agriculture, under supervision of the Veterinary Services (Agreement A44565).

**Detection of anti-Tk Antibodies in the Sera of Immunized Rats.** Blood samples from immunized rats were taken at an eye sinus site before immunization (preimmune serum) and after immunization (immune serum). Sera were obtained and kept frozen at −80°C, before being assayed for the presence of anti-Tk antibodies by ELISA. Ninety-six-well plates (Nunc) were coated with the structure GlcNAcβ₁-₄GlcNAc-Sp⁻³-PAA at 10 μg/ml in 0.05 mol/l sodium carbonate buffer (pH 9.6) overnight at 4°C. Plates were then incubated with 3% BSA containing PBS. Between all steps, plates were washed three times with PBS containing 0.1% Tween 20. Serum samples were then added, diluted 1:50 in PBS containing 0.3% BSA, and incubated for 1.5 h at 37°C. After being washed, plates were incubated with anti-rat IgG alkaline phosphatase conjugate (Sigma) for 1 h at 37°C. Finally, reactions were developed by incubation with p-nitrophenylphosphate substrate (Sigma) diluted at 1 mg/ml in 0.05 mol/l sodium carbonate buffer and read at 405 nm.

**Statistical Analysis.** In the survival experiment, the significance of the observed differences between survival rates was determined by the log-rank test.

**RESULTS**

**Expression of the Epitope Recognized by mAb LM389 in Normal Tissues and Colorectal Carcinomas.** Antibody LM389 was selected for its ability to selectively agglutinate erythrocytes with a Tk phenotype. On the basis of its serological reactivity, it can be considered as a specific anti-Tk reagent because it reacts exclusively with endo-β-galactosidase-treated erythrocytes and in *vivo*-modified Tk red cells (24). To know if the epitope recognized could be present on other cell types, the antibody was tested by immunohistochemistry on normal tissues. The results are summarized in Table 1. Reactivity was mainly visible on epithelial cells of the digestive tract. Thus, the esophagus epithelium, the stomach, the small and large intestines, as well as the exocrine pancreas were stained by the anti-Tk reagent. In addition, epithelial cells of the trachea and to a lesser extent of the urinary bladder were also stained. However, in all of these tissues, the staining was intracellular, mostly limited to a supranuclear area typical of the Golgi region. The strongest staining was observed in the colon, as depicted on Fig. 1A. None of the other tissues tested showed reactivity. The binding of the anti-Tk mAb was also tested in normal rat tissues. Strikingly, the staining of tissues from this animal species paralleled that of human tissues and was similarly restricted to a supranuclear location, suggesting that the carbohydrate epitope recognized could correspond to a core structure masked at the cell surface by further elongation of the glycan chain. This observation prompted us to look for the presence of the Tk epitope in adenomas and colorectal carcinomas. As shown in Table 2, it appeared that none of the 16 adenomas tested reacted with mAb LM389. However, strong reactivity could be detected in the case of carcinomas. It was no longer restricted to the Golgi area because the strongest staining was observed on cell membranes and secretory material (Fig. 1, B and C). Of 56 tumors, 13 showed a uniform strong staining of the carcinoma cells. The remaining tumors were separated into three groups: those with large areas, estimated between 25 and 75% of the cancer cells, presenting a strong staining; those where the positive areas were restricted to 5–25% of the cancer cells; and finally, those that were either completely devoid of positive cells or that contained only some rare such cells. Altogether, the positive cases represented 71% of the total. Yet, the two first groups with large positive areas represented 48% of the cases. No association could be observed with either the location of the primary tumor or the Tumor-Node-Metastasis staging. In transitional mucosa, staining was similar to that in normal mucosa, which is restricted to the supranuclear area of epithelial cells. To examine whether the presence of the antigen could be associated with a more or less metastatic phenotype, we looked for its presence on couples of primary and metastatic tumor tissues from eight patients. The results are summarized on Table 3. The primary carcinomas were from the colon, except for one case, where it originated...
from the rectum. Metastasis were mainly from the liver, except for one case of mesenteric lymph node metastasis and one case of peritoneal cavity metastasis. All cases presenting with staining of the primary tumor showed the same degree of staining in the metastatic tissue, whereas metastatic tissue derived from primary tumors that were not stained did not show any labeling either. These results tend to indicate that no selection, either positive or negative, of the antigen expression occurred during the process of metastasis.

Expression of the Antigen on Human and Murine Cell Lines. To determine the biochemical mechanisms responsible for the abnormal presence of Tk-reactive epitopes at the surface of colon cancer cells, to test the biological roles of this newly acquired cell surface antigen, and to study its potential interest as a target of immunotherapy, it would be desirable to obtain cell lines that express or lack the antigen. For this aim, we screened a series of human and murine cell lines. Ten human cell lines of colorectal origin were tested, i.e., Colo205, HT29, HRT18, HCT-GEO, LoVo, LS174T, SW620, SW707, SW1116, and ALT-1. Of these, only 3 cell lines, LS174T, SW707, and SW1116, contained a small subpopulation of positive cells (<5%). The other cell lines were entirely negative. Tk-reactive and unreactive cells were established from the SW707 cell line by two rounds of enrichment using magnetic beads, followed by limiting dilution cloning (Fig. 2). A cell surface glycan profile of the clones thus obtained was defined using a panel of lectins and anticarbohydrate mAbs. This allowed us to reveal the presence of two types of Tk-positive and Tk-negative clones that could be distinguished on the basis of their reactivities with these reagents (not shown). Of the reagents tested, the only one that gave a reactivity correlating with that of the anti-Tk was the anti-STn mAb (TKH2). The reactivity of one example of each type of clone with the anti-Tk and anti-STn mAbs is

Table 2 Reactivity of mAb LM389 with colorectal carcinomas and adenomas

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>% total number</th>
<th>% positive tumor cells</th>
</tr>
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<tbody>
<tr>
<td>Carcinomas</td>
<td>16</td>
<td>28.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>23.2</td>
<td>5–25</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>25.0</td>
<td>25–75</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>23.2</td>
<td>75–100</td>
</tr>
<tr>
<td>Adenomas</td>
<td>16</td>
<td>100</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* Antibody reactivity was tested on fixed tissue sections using a biotin and avidin-peroxidase assay. Carcinomas were divided into four groups according to the percentage of positive cells. They were scored as negative below 5% positive cells, as weakly positive below 25% positive cells, as moderately positive below 75% positive cells, and as strongly positive from 75 to 100% positive cells.

Table 3 Comparative LM389 reactivity between the primary tumor and a metastasis from individual patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Origin of primary tumor</th>
<th>% positive tumor cells</th>
<th>Site of metastasis</th>
<th>% positive tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colon</td>
<td>25–75</td>
<td>Lymph node</td>
<td>25–75</td>
</tr>
<tr>
<td>2</td>
<td>Colon</td>
<td>25–75</td>
<td>Liver</td>
<td>25–75</td>
</tr>
<tr>
<td>3</td>
<td>Rectum</td>
<td>25–75</td>
<td>Liver</td>
<td>25–75</td>
</tr>
<tr>
<td>4</td>
<td>Colon</td>
<td>5–25</td>
<td>Liver</td>
<td>5–25</td>
</tr>
<tr>
<td>5</td>
<td>Colon</td>
<td>&lt;5</td>
<td>Liver</td>
<td>&lt;5</td>
</tr>
<tr>
<td>6</td>
<td>Colon</td>
<td>&lt;5</td>
<td>Liver</td>
<td>&lt;5</td>
</tr>
<tr>
<td>7</td>
<td>Colon</td>
<td>&lt;5</td>
<td>Liver</td>
<td>&lt;5</td>
</tr>
<tr>
<td>8</td>
<td>Colon</td>
<td>&lt;5</td>
<td>Peritoneum</td>
<td>&lt;5</td>
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</tbody>
</table>

* Antibody reactivity was tested on fixed tissue sections using a biotin and avidin-peroxidase assay. Reactivity was scored as in Table 2.
given in Fig. 3. As mentioned above, a small subset of Tk-positive cells were visible in the LS174T cell line. In addition, STn-positive and -negative clones (LSC and LSB) were obtained previously by Ogata et al. (25) using a selection process similar to that used here to select SW707 Tk-positive or -negative cells. We thus tested the reactivity of the LSC and LSB cells with the anti-Tk mAb (TKH2). As shown on Fig. 2, the STn-positive clone LSC was also Tk positive, whereas the STn-negative clone LSB was almost completely Tk negative, substantiating the relationship between the expression of the two antigens STn and Tk on human colorectal carcinoma cell lines.

Twenty-four animal cell lines were equally tested for their expression of the Tk antigen. Nine cell lines were from rat tumors, and 15 were from mice. They represented tumors from various origins. Of the 24 cell lines, only the PROb cells were strongly positive with the anti-Tk mAb upon fluorescence-activated cell sorter analysis. Another cell line, also tumorigenic in BDIX rats, was tested. These cells, called A15A5, originate from a glioma and were completely unreactive with mAb LM389 (Fig. 2). Total cell extracts from the four cell lines LSB, LSC, A15A5, and PROb were submitted to Western blotting (Fig. 4). MAb LM389 stained a major broad band centered around $M_r$ 150,000 on the rat and human Tk-positive cells LSC and PROb, respectively. Minor bands were also visible on these two cell lines, at above $M_r$ 200,000, $M_r$ 90,000, and $M_r$ 65,000. In LSB extracts, a weaker band at $M_r$ 150,000 was visible, indicating that a small amount of Tk-reactive material is present on a glycoprotein from this clone. However, no reactive band could be detected on the rat glioma A15A5 cells. Although the precise nature of the labeled glycoproteins is not determined as yet, the human and rat positive and negative cells are expected to provide useful tools for further study of the Tk antigen.

The epitope recognized by mAb LM389 is present on O-Glycans. To define the type of glycan that carries the Tk reactivity on colonic cancer cells, tumor sections were incubated with of O-sgp. This proteolytic enzyme cleaves proteins that are glycosylated on serine or threonine residues. Treated sections were then tested for their reactivity with the anti-Tk mAb. As shown on Fig. 1, C and D, the untreated control section is strongly reactive, whereas the subsequent serial section treated with the enzyme shows an almost complete disappearance of the mAb staining, suggesting that the Tk epitopes are carried by O-glycans. To confirm this observation, LSC cells were cultivated in the presence of benzyl-2-acetamido-2-deoxy-$\alpha$-D-galactopyranoside, an inhibitor of O-glycosylation. As shown on Fig. 5, A.

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**Fig. 2.** Cytofluorimetric analysis of Tk antigen expression on human and rat cells using mAb LM389. Shown are examples of clones derived from the SW707 human colon carcinoma cell line, clones C8 and D5; clones LSC and LSB derived from the LS174T human colon carcinoma cell line; clone PROb derived from the DHDK12 rat colon carcinoma cell line and rat A15A5 glioma cell line. The log of fluorescence intensities in arbitrary units is plotted against cell number. Fluorescence intensities from cells incubated in the presence of mAb LM389 (black lines) are superimposed on fluorescence intensities from control cells incubated with the FITC antimusese immunoglobulin only (gray lines).

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**Fig. 3.** Cytofluorimetric analysis of cell surface glycosylation using FITC-labeled lectins and mAbs. Clonal cells were derived from SW707 after selection using mAb LM389 and magnetic beads followed by limiting dilution. Shown are examples of two clones labeled by mAb LM389 (C6 and F2) and of two unlabeled clones (B2 and G6). Mean intensities of fluorescence obtained with each reagent are given in arbitrary units. Control corresponds to the mean fluorescence intensity of cells incubated with the FITC antimusese immunoglobulin only.

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**Fig. 4.** Western blot analysis of total cell extracts using mAb LM389. Extracts were from clones LSC and LSB derived from the LS174T human colon carcinoma cell line and clone PROb derived from the DHDK12 rat colon carcinoma cell line and rat A15A5 glioma cell line. Molecular weight markers (in thousands; $kD_a$) are indicated.
immunoglobulin only (gray lines) control cells incubated with the FITC antimouse are superimposed on fluorescence intensities from

Fig. 6. Adsorption of mAb LM389 on oligosaccharides coupled to silica beads (SYNSORBs). mAb LM389 was incubated on beads substituted with three different oligosaccharides, the structures of which are indicated, and substituted with uncoupled beads for use as a nonspecific adsorption control. Reactivity of the supernatants was then tested by flow cytometry on PROb rat cells. The percentage of adsorption was calculated from the ratio of mean fluorescence from supernatants incubated on oligosaccharide-substituted beads to that from the nonspecific adsorption control. Values represent means of four independent experiments.

Fig. 5. Determination of the type of glycan-carrying Tk epitopes on LSC human colon carcinoma cells. A, cells were treated or not (Control) with the O-sgp, and their reactivities with mAb LM389 were tested by flow cytometry. B, after SDS-PAGE and blotting on nitrocellulose, cell extracts were treated or not (Control) with O-sgp and simultaneously immunostained with mAb LM389 and an anti-actin mAb. The major band stained by LM389 is visible at about M, 150,000, and the M, 42,000 band corresponds to actin. C, cells were treated with the N-glycosylation inhibitor DMJ, and their reactivities with mAb LM389 and the lectin L-PHA, which detects the β1-6 branch of N-glycans, were tested by flow cytometry. The log of fluorescence intensities in arbitrary units is plotted against cell number. Fluorescence intensities from cells incubated in the presence of mAb LM389 and L-PHA (black lines) are superimposed on fluorescence intensities from control cells incubated with the FITC antimouse immunoglobulin only (gray lines).

The Tk reactivity of treated cells was greatly reduced compared with that of control cells cultured in standard conditions. Furthermore, total LSC protein extracts blotted on nitrocellulose were treated with O-sgp. This treatment strongly decreased the staining of the major Tk-reactive band at M, 150,000. Yet as expected, it had no effect on the antiactin staining (Fig. 5B). These results confirm that the Tk epitope is carried by O-glycans. To determine whether it could also be carried by N-glycans, LSC cells were cultivated in the presence of DMJ, an inhibitor of the maturation of N-glycosylation. This inhibitor was preferred over the commonly used tunicamycin because the latter is highly toxic to cells and could inhibit the transport of glycoproteins to the cell surface. As depicted on Fig. 5C, DMJ-treated cells had a greatly reduced reactivity with L-PHA, showing that maturation of N-glycans was effectively inhibited. However, the treatment had no effect on the binding of the anti-Tk mAb, indicating that the epitope is not carried on N-glycans.

Determination of the Epitope Recognized by mAb LM389. To define more precisely the epitope recognized by the anti-Tk mAb, its reactivity was tested against a series of synthetic carbohydrates. In a first set of experiments, the antibody was adsorbed on immobilized oligosaccharides (SYNSORBs), and the reactivity remaining in the supernatant was tested by flow cytometry on the PROb rat Tk-positive cells. Of the three immobilized oligosaccharides tested, only one of them, the trisaccharide GlcNAcβ1–3Galβ1–4Glc, completely adsorbed the antibody. At variance, the two oligosaccharides terminated with galactose residues did not adsorb significant amounts of the antibody (Fig. 6). This result is in accordance with the fact that the antibody was raised and selected against erythrocytes treated with an endo-β-galactosidase, which is expected to unmask GlcNAc residues. Given this first result, in a second set of experiments, the binding of mAb LM389 to various synthetic neoglycoconjugates, most with terminal GlcNAc units, was tested by ELISA. Strong binding was observed only on the tetrasaccharide GlcNAcβ1–6(GlcNAcβ1–3)Galβ1–4Glc and on chitobiose (GlcNAcβ1–4GlcNAc). To obtain a more precise estimate of the anti-Tk antibody reactivity with synthetic carbohydrates, the ability of oligosaccharides, either as monomers or as polyacrylamide conjugates, to inhibit its binding to chitobiose was quantified. Results are summarized in Table 4. The two best inhibitors that could be tested as monomers were the same two oligosaccharides to which the antibody strongly bound in the direct binding assay. The inhibition curves given by these two compounds are shown on Fig. 7. Although the amounts of each oligosaccharide necessary for 50% inhibition are not very different, the slope of the inhibition curve is much steeper in case of the tetrasaccharide GlcNAcβ1–6(GlcNAcβ1–3)Galβ1–4Glc than in case of the disaccharide GlcNAcβ1–4GlcNAc, indicating a stronger affinity for the former. Two other substances were quite strongly inhibitory; they consisted, respectively, of a single GlcNAc unit or of the trisaccharide GlcNAcβ1–6(GlcNAcβ1–3)GalNAc attached to an aglycon part. It should be noted that the trisaccharide GlcNAcβ1–3Galβ1–4Glc, which adsorbed completely the anti-Tk mAb when linked to a solid support (Fig. 6), was only a weak inhibitor in a monomeric form, indicating that anticarbohydrate antibodies can be adsorbed on insolubilized carbohydrates for which they only have a weak affinity. Among the polyacrylamide conjugates tested, only a conjugate containing the GlcNAcβ1–4GlcNAc was strongly inhibitory. In this case, the importance of the spacer between the carbohydrate and polyacrylamide is illustrated by the weak inhibitory potency of the same disaccharide attached via the short spacer Sp1 compared with that with the longer spacer Sp2. Overall, it should be noted that the strong inhibitors have in common either two terminal GlcNAc residues forming a branch or a single terminal GlcNAc along with a second N-acetyl group within the spacer or the aglycon part. In the latter case, it is likely that this
Table 4  Inhibition of mAb LM389 binding to GlcNAcβ1–4GlcNAcβ1–Sp3–PAA by synthetic oligosaccharides

<table>
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<th>Oligosaccharide</th>
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<tr>
<td>Galβ1–4Glcβ1–O(CH2)3NH2</td>
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<tr>
<td>GlcNAcβ1–4Glcβ1–O(CH2)3NH2</td>
<td>150</td>
</tr>
<tr>
<td>GlcNAcβ1–4Glcβ1–O(CH2)2NHAc</td>
<td>170</td>
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<tr>
<td>GlcNAcβ1–4Glcβ1–O(CH2)2NH2</td>
<td>190</td>
</tr>
<tr>
<td>GlcNAcβ1–4Glcβ1–O(CH2)2NHAc</td>
<td>80</td>
</tr>
</tbody>
</table>

*Binding of mAb LM389 to GlcNAcβ1–4GlcNAcβ1–Sp3–PAA was measured by ELISA. Binding could also be obtained using GlcNAcβ1–4GlcNAcβ1–Sp3–PAA but not using GlcNAcβ1–4GlcNAcβ1–PAA. GA1β1–3Galβ1–4GlcNAcβ1–PAA and GlcNAcβ1–4GlcNAcβ1–Sp3–PAA were assayed either as monomers or as multivalent conjugates to PAA.

Second N-acetyl group imitates the second GlcNAc residue of the branched structure. Therefore, it is expected that the natural antigen recognized by the antibody should have two nonreducing GlcNAc units forming a branch.

Protection against Tumor by Immunization with Tk-positive Erythrocytes. To test the effect on tumor growth of immunization against Tk epitopes, rats were immunized using human erythrocytes as a source of Tk antigen. Native human erythrocytes are not agglutinated at all by mAb LM389. Endo-β-galactosidase-treated red cells are weakly agglutinated. A strong agglutination is obtained by treating red cells with both papain and endo-β-galactosidase, but papain treatment alone does not allow agglutination by the antibody (24).

Thus, rats were immunized against either native or papain-treated erythrocytes, which will not present Tk epitopes, and against endo-β-galactosidase-treated erythrocytes or papain/endo-β-galactosidase-modified erythrocytes to obtain cells that weakly or strongly, respectively, express Tk epitopes. In the first experiment, animals received i.v. injections of red cells and were challenged s.c. either with the strongly positive PROb cells or with A15A5 Tk-negative cells. As shown on Fig. 8, fast PROb tumor growth was observed in rats immunized with native red cells. A slight tumor growth delay was visible in rats immunized with endo-β-galactosidase-treated erythrocytes. However, a much stronger growth delay was observed in rats immunized with the strongly Tk-positive erythrocytes obtained by treatment with the two enzymes. Indeed, in this last case, tumor growth was completely absent in two of six animals, strongly reduced in three other animals, and only one single animal presented with a fast-growing tumor. At variance with this result, fast tumor growth was observed in all animals challenged with the Tk-negative tumor cells A15A5, irrespective of whether they were immunized with Tk-negative or with Tk-strongly positive erythrocytes. The presence of antibodies directed against the Tk cross-reactive disaccharide GlcNAcβ1–4GlcNAc, coupled to PAA, was determined by ELISA. In this preliminary set of experiments, this disaccharide was used in place of the tetrasaccharide GlcNAcβ1–6(GlcNAcβ1–3)Galβ1–4Glc because too small amounts of the latter were available. As shown on Fig. 8, sera from rats immunized with native red cells did not show a higher reactivity toward GlcNAcβ1–4GlcNAc than did preimmune sera. In contrast, a much stronger reactivity was observed in the serum of five of six rats immunized with papain/endo-β-galactosidase-modified red cells. Only one immune serum sample in this group had not enhanced its reactivity. It corresponds to the serum from the only rat that presented a fast-growing tumor, strongly suggesting a relationship between the presence of antibodies able to recognize GlcNAcβ1–4GlcNAc and tumor growth delay. No increase in reactivity against this disaccharide was visible in the immune sera from rats that had received either papain-treated or endo-β-galactosidase-treated erythrocytes (data not shown). In a second experiment, rats received i.p. injections of red cells and were challenged i.p. with either PROb or A15A5 cells, and their survival was monitored (Fig. 9). Rats that received the Tk-negative cells had a similar median survival whether they had been immunized with Tk-positive or with Tk-negative erythrocytes (53 days versus 55 days). However, rats that received the Tk-positive tumor survived much longer after being immunized against Tk-positive red cells than after being immunized against native red cells (79 days versus >187 days), and this difference was highly significant (P < 0.002). Thus, immunization against the Tk antigen can confer specific protection toward a tumor expressing the antigen.

DISCUSSION

The Tk antigen is serologically defined as a polyagglutination antigen (20). In the present study, we have observed that an antibody raised against Tk-positive red cells strongly stains a significant proportion of colorectal carcinomas cells, whereas it only stains normal epithelial digestive cells at the Golgi level. In this respect, the Tk antigen is very similar to the Tn and Tn antigens and can thus be considered as a new colon cancer associated carbohydrate antigen. Nevertheless, it differs from these antigens and from the STn antigen because it was neither detected in adenomas nor in transitional mucosa (14). A strong cell surface expression of the Tk antigen could only be detected in carcinomas. Thus, the antigen appears to be relatively cancer specific, yet not present at early stages of the cancer progression. On colorectal cancer cells, Tk antigen is carried on O-glycans because the immunoreactivity could be destroyed by treatment with O-sgp. Its presence on glycolipids was not examined.
expressed on most cell lines tested, and only small subpopulations with two GlcNAc units. Its conformation could mimic that of a branched structure terminated by the epitope recognized by mAb LM389 on colon carcinoma cells. N-core of O-expected to be present on A–F growth was monitored, each line representing and E endo- and C papain and Sp 3-PAA were detected by ELISA (10, 6)). After the last injection, rats received s.c. 1 × 10^9 Tk-positive PROb (A, B, C, D, G, and H) or Tk-negative A15A5 cells (E and F). Tumor growth was monitored, each line representing growth in a single animal (A–F), and serum antibody reactivities toward GlcNAcβ1–4GlcNAcβ1–Sp-PAA were detected by ELISA (G and H). Preimmune (□) and immune (●) individual rat sera were tested at a 1:50 dilution.

Therefore, it cannot be excluded at present. Earlier studies tentatively characterized its structure on the erythrocyte membrane as GlcNAcβ1–6(GlcNAcβ1–3)Gal (22). This is in accordance with the specificity of the anti-Tk mAb LM389 because structures terminated with two branched GlcNAc residues, such as GlcNAcβ1–6(GlcNAcβ1–3)GallNac, were among the best inhibitors. GlcNAc-terminated antigens have been found previously in early stage development (28, 29) as well as on normal and neoplastic gastric tissue (30). Such structures are typically present in O-glycans but are normally elongated and terminated by galactose and Fuc or sialic acid residues (31). Because their biosynthesis takes place in the Golgi apparatus, it would explain the reactivity of the antibody with a supranuclear area of normal cells, the Tk antigen corresponding to a synthesis intermediate. In colorectal carcinomas, O-glycan chains are much shorter than in the normal mucosa (32). Defects in the elongation of these chains could lead to the expression at the cell surface of precursor structures like the Tk antigen. Surprisingly, in addition to the branched GlcNAc-terminated oligosaccharides, the antibody showed a strong reaction with the disaccharide GlcNAcβ1–4GlcNAc. This disaccharide is not expected to be present on O-glycans. It corresponds to the innermost core of N-glycans (33). It is thus unlikely that this disaccharide would be the epitope recognized by mAb LM389 on colon carcinoma cells. Its conformation could mimic that of a branched structure terminated with two GlcNAc units.

Similar to the Tn and sialyl Tn antigens, Tk antigen was not expressed on most cell lines tested, and only small subpopulations expressed the antigen in the few positive cell lines. It could explain why this antigen has not been described earlier as a tumor-associated antigen, because most mAbs defining tumor-associated antigens have been raised using cell lines as the immunogen. Surprisingly, the clones that uniformly expressed the Tk antigen also expressed STn. Because both antigens result from alterations of O-glycans biosynthesis, some of the underlying molecular defects responsible for their expression might be common. Two distinct molecular mechanisms can account for the expression of the STn antigen. Because it is present in an acetylated form in the normal colonic mucosa, a loss of acetylgalactosaminyltransferase activity is sufficient to reveal the cancer-associated epitope (15, 16). This simple molecular defect cannot lead to expression of the Tk antigen because the latter is not sialylated, but it could occur early during carcinogenesis. At variance, in LSC cells, it has been established that more drastic defects of O-glycan biosynthesis were responsible for the synthesis of the short STn chains (34). These cells, unlike LSB cells that do not present either STn or Tk epitopes, lack detectable core 1 galactosyltransferase and all N-acetylgalcosaminyltransferases required to extend O-glycan chains. The presence of Tk epitopes on LSC O-glycan chains suggests that at least residual N-acetylgalcosaminyltransferase activities are present in these cells. In absence of competition with core 1 galactosyltransferase, these residual enzymatic activities could be sufficient to synthesize the Tk antigen. Such major defects in O-glycan biosynthesis would only be found at later stages of the carcinogenesis process, explaining why the Tk reactivity was restricted to carcinomas.

Recent studies of cancer immunotherapy focused on cancer-associated peptide targets recognized by T cells (35, 36). However, the efficacy of passive immunotherapy using antibodies was documented recently for colorectal cancer patients (37). Moreover, active immunization against carbohydrate antigens such as the T and sialyl Tn antigens yielded encouraging results in association with the development of antibody responses (19, 38). The absence of Tk antigen at the cell surface of normal tissues, together with the existence of natural antibodies, suggested that this antigen could be a new interesting target of colon cancer immunotherapy. To test this possibility, we searched for an appropriate animal model. Two cell lines from BDIX rats, PROb and A15A5, respectively, were found that express or are devoid of the antigen. The distribution of Tk epitopes in the rat normal tissues was similar to that in human normal tissues. This experimental model can thus be considered as a valuable tool to assay the usefulness of the Tk antigen as a target of immunotherapy and to set up immunization protocols. Preliminary experiments using endo-β-galactosidase-treated erythrocytes as the immunogen, showed that growth of the Tk-positive tumor cells but not of Tk-negative tumor...
cells was retarded or completely abolished in immunized animals. This antitumor effect was related to the presence of antibodies cross-reactive with the antigen, indicating that indeed this new carcinoma-associated antigen could be an interesting target of immunotherapy. The antibody reactivities raised by the immunization were rather low. It should be noted that they were probably not tested against the optimal synthetic antigen. In addition, immunizations were performed in the absence of adjuvant. It can be expected that the use of adjuvants of synthetic oligosaccharides such as GlcNAcβ1→6(GlcNAcβ1→3)Galβ1→4Glc or GlcNAcβ1→6(GlcNAcβ1→3)GalNAc, coupled to a potent immunogenic carrier, should improve the immune response. The availability of the animal model will allow testing of such approaches. In addition, the availability of Tk-positive human cells, such as LCS- or the SW707-derived clones, will allow evaluation of the antibody responses against the cellular antigen and not only against synthetic oligosaccharides. These cell lines should also be useful models to define the molecular mechanisms responsible for the expression of the antigen at the cell surface and to define its potential biological role.

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Tk, a New Colon Tumor-associated Antigen Resulting from Altered O-Glycosylation

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