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# A rat model to study the role of STn antigen in colon cancer

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**Expression of the mucin-associated sialyl-Tn (STn) antigen has been associated with a decreased survival in patients with colorectal, gastric, and ovarian cancer. To better understand the role of STn antigen in tumor biology, we developed STn(+) (called LP) and STn(−) (called LN) clonal cell lines from a parental metastatic rat colon carcinoma cell line (LMCR). Both derivative cell lines exhibited identical proliferation rates *in vitro*. LP cells strongly expressed STn antigen both *in vitro* and *in vivo*, and were poorly tumorigenic when given to syngeneic rats. LN cells did not express STn antigen *in vitro*, but as *in vivo* tumors these cells rapidly acquired STn expression, readily formed tumors, and were highly lethal. When rats were given an otherwise lethal inoculum of *i.p.* LN cells, pre-immunization with synthetic STn antigen conjugated to keyhole limpet hemocyanin (STn-KLH) resulted in a 60% survival rate. When LN cells were injected subcutaneously in the presence of STn-KLH-sensitized lymphocytes, tumor growth was decreased. Distribution of STn antigen in normal organs of host rats is quite similar to that of humans. This model mimics human disease and should facilitate studies of mucin-associated antigens in tumor biology and the development of immunotherapeutic agents based on mucin-related antigens.**

***Keywords:*** **sialyl-Tn, mucin, immunotherapy, colon cancer**

***Abbreviations:*** **Sia, sialic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; ser/thr, serine/threonine.**

## Introduction

Sialyl-Tn (STn) is a mucin-associated carbohydrate antigen (Sia$\alpha$2,6GalNAc-O-ser/thr) that has attracted interest as a clinically important tumor antigen for several reasons. First, STn expression is rarely found in normal tissues. Goblet cells of the small intestine, gastric parietal cells, testicular Leydig cells, endometrial cells in the secretory phase of the menstrual cycle, some endothelial cells (particularly those of gastrointestinal mucosa), and conjunctival cells are among the only cells in the body that express STn [1–5]. In contrast, most types of adenocarcinomas express STn, including colorectal, gastric, pancreatic, breast, ovarian, and lung [1–4,6–12]. Thus, STn is a rather specific and sensitive diagnostic marker of adenocarcinoma. Second, STn may be a useful marker of prognosis since expression of STn in tumor tissues and sera has been associated with a poor prognosis for patients with colorectal [13], gastric [3,14,15], and ovarian [16] cancer, and may help predict the effect of adjuvant chemotherapy in patients with node-positive breast cancer [17]. Third, STn antigen expression correlates with premalignant and malignant progression in the gastrointestinal tract. On the one hand, STn expression can be detected in premalignant tissues such as adenomatous polyps [18], chronic ulcerative colitis [19,20], and intestinal metaplasia [3,4,8,9]. On the other hand, STn may also be a marker of progression toward a metastatic phenotype since metastases of colorectal cancer often express STn more than primary tumors [21]. These observations suggest that STn antigen may play a role in the biological behavior of cancer cells.

The potential for using STn antigen for immunotherapy has been explored [22]. In mice, immunization with STn and Tn vaccines protected mice from a subsequent challenge with syngeneic cancer cells expressing these antigens [23,24]. In patients with metastatic adenocarcinoma undergoing active specific immunotherapy with synthetic STn conjugated to keyhole limpet hemocyanin (STn-KLH; THERATOPE), antibodies against STn correlated with survival [25]. Moreover, patients with breast and ovarian adenocarcinoma who undergo

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autologous stem cell rescue and are then vaccinated with STn-KLH mount strong cellular and humoral immunity against the vaccine [26] and show a tendency toward lower risk of tumor relapse and death [27].

To more clearly elucidate the role of this antigen *in vivo*, however, it would be important to develop an appropriate animal model. A rat colon cancer has been described in which the expression of blood group-related oligosaccharides correlated with progressive tumor growth [28,29]. A derivative of this cell line with high metastatic potential was developed and termed LMCR [30]. We noted that LMCR cells expressed high levels of STn antigen. Using this cell line, we cloned an STn(+) and STn(−) cell line and report herein their *in vitro* and *in vivo* characteristics, tumorigenicity and potential utility for testing STn-based immunotherapy.

## Materials and methods

### Establishment of STn(+) and STn(−) clones

The parental LMCR rat colon cancer cell line used in these studies was derived originally from the K12/TRb rat colon cancer cell line (31; kindly supplied by Dr. F. Martin, University of Dijon, France). LMCR cells were established by intrasplenic injection of tumor cells, followed by cell culture of resulting pulmonary nodules, repeating this process for five cycles [30]. We observed that parental LMCR cells heterogeneously expressed STn (see Results). STn(+) and STn(−) cells were then cloned from LMCR cells using a two-step method in which cells were first enriched for STn expression and then cloned by limiting dilution; the same method used to isolate STn(+) and STn(−) clones from a human colon cancer cell line [32].

#### *Step 1—Enrichment of STn(+) cells*

After trypsinization, $5 \times 10^6$ LMCR cells were suspended in 1 ml of mouse monoclonal antibody TKH2 (hybridoma culture supernatant). Following a 30 minute incubation at 4°C, cells were washed twice with DMEM by centrifugation. The cells were then resuspended in DMEM and magnetic polystyrene beads coated with sheep anti-mouse IgG antibody (Dynabeads M-450, DYNAL INC, Great Neck, NY) were added, using a bead:cell ratio of 3:10. The suspension was again incubated at 4°C for 5 min. According to the manufacturer's protocol, cells which bound to the beads were collected by a magnet and washed with DMEM. The collected cells together with the bound beads were placed in culture and incubated in DMEM supplemented with 10% fetal calf serum and antibiotics under standard conditions. Beads were later removed when the cells were trypsinized for expansion.

#### *Step 2—Cloning of STn(+) and STn(−) cells*

Cell cultures enriched for STn expression in Step 1 were trypsinized and the concentration of cells was adjusted so that each well of a 96-well plate received one cell. After 24 hours, wells with a single cell were marked. Single cell colonies were grown, expanded, and screened for STn antigen expression by immunocytochemistry on cells grown in chamber slides (Nunc, Inc. Naperville, IL) using MAb TKH2. One STn-negative and one STn-positive clone was selected and a second round of limiting dilution cloning was performed to ensure clonality. The STn-negative clone was named LMCR(−) (LN for short) and the STn-positive clone was named LMCR(+) (LP for short).

### *Cell culture and animals*

LMCR cells and its derivatives were fed with DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 $\mu$g/ml streptomycin and incubated at 37°C in 7.5% $CO_2$. Male BDIX rats, 4–6 weeks old, were obtained from Charles River Laboratories through the National Cancer Institute. All studies were approved by our Institutional Animal Care and Use Committee.

### *Growth curves and lethality*

*In vitro* growth rates of LP and LN cells were determined by seeding $2 \times 10^5$ cells in duplicate T-25 flasks on day 0 and counting the cell numbers on day 2, 4, 7, and 9. Medium was changed every 3–4 days. *In vivo* growth of cells was examined by measuring the size of tumors that developed following subcutaneous injection of each cell line into syngeneic host BDIX rats. Tumor size was expressed as a volume calculated by the equation: $V = a \times b^2/2$, where $a$ and $b$ represent the long and short axis, respectively. Lethality of the cell lines in syngeneic animals was determined by daily monitoring of survival of rats given intraperitoneal injection of tumor cells at different doses.

### *Detection of carbohydrate antigens in cell lines and tumors*

Several mucin-associated antigens were examined in cell cultures and tumors from LN and LP cells by immunohistochemistry. Mouse monoclonal antibodies 1E3 (anti-Tn), TKH2 (anti-STn), SNH3 (anti-$SLe^x$) were generous gifts of Professor Sen-itiroh Hakomori (The Biomembrane Institute, Seattle, WA). Sialyl-$Le^a$ was detected with MAb CA19-9 (hybridoma obtained from American Type Culture Collection, Rockville, MD). The Thomsen-Friedenreich (TF) antigen and blood group H antigen were analyzed using biotinylated peanut agglutinin (PNA) and biotinylated ulex europaeus agglutinin-1 (UEA-1) lectins, respectively (Sigma Chemical Co., St. Louis, MO). Mab OX-18 directed against rat MHC Class I antigen was obtained from Pharmingen (San Diego, CA). Cells grown as monolayers on chamber slides were fixed with methanol:acetic acid (3:1). Formalin-fixed, paraffin-embedded tumors were sectioned (4 $\mu$m thickness) and stained with the monoclonal antibodies or lectins as previously described [32].

*Immunization with synthetic carbohydrate antigens*

The STn disaccharide was conjugated through a two carbon crotyl linker to keyhole limpet hemocyanin (STn-KLH) [33]. This reagent at a dose of 1 $\mu$g or 100 $\mu$g was emulsified with Ribi Adjuvant System (Ribi Immunochemicals, Hamilton, MT) and injected subcutaneously. Control groups received either KLH (1 $\mu$g) alone, or immunization with STn-KLH (1 $\mu$g) followed by a boost with the closely related Tn-KLH (1 $\mu$g) ("cross-over group").

*Humoral immune response to STn-KLH*

To determine whether animals mounted an antibody response to STn-KLH, rats were immunized with STn-KLH at either 1 $\mu$g or 100 $\mu$g, boosted twice at two-week intervals, and serum was collected after each immunization. IgG and IgM responses were measured by ELISA.

*Winn-type assay*

The effect of STn-sensitized lymphocytes was tested in a Winn-type assay. Animals were immunized with either PBS, KLH (1 $\mu$g), STn-KLH (1 $\mu$g), or STn-KLH (100 $\mu$g), and then boosted with the same reagent on Day 14 and Day 28. On Day 36, spleens were harvested and lymphocytes prepared. Splenic lymphocytes ($20 \times 10^6$) were mixed with LN cells ($1 \times 10^6$) and inoculated subcutaneously. Tumor growth was measured serially, and tumor volumes calculated as above.

## Results

*In vitro* characteristics of LP and LN cells

*Establishment of cell lines based on STn antigen expression*

The K12/TRb cells weakly express STn antigen (Figure 1A), whereas LMCR cells which have undergone five cycles of intrasplenic passage followed by culturing lung metastases, demonstrated a marked increase in STn expression (Figure 1B). However, not all of the LMCR cells expressed STn antigen. Therefore, LMCR cells were enriched for STn expression using a magnetic bead approach, and then cloned by limiting dilution. In monolayer cultures, the STn-positive clone, LP, uniformly expressed STn antigen (Figure 1C) whereas the STn-negative clone, LN, completely lacked STn expression (Figure 1D).

*Growth rate in vitro*

The *in vitro* growth rate of the two cell lines was nearly identical, with a doubling time of about 20 hours (Figure 2).

*Expression of carbohydrate antigens in vitro*

The expression of several mucin-associated antigens by LP and LN cells is shown in Table 1. In monolayer culture, LP cells expressed both STn and its precursor Tn antigens, whereas LN cells did not express STn or Tn antigen. Neither clone expressed TF, or the complex carbohydrate antigens sialyl-Le$^a$ or sialyl-Le$^x$, but both cell lines expressed blood group H antigen detected by UEA-1. The two cell lines did not differ in their expression of rat MHC Class I antigen.

*In vivo* characteristics of LP and LN cells

*Tumorigenicity of LN and LP cells*

*In vivo* growth properties of the cell lines was first investigated by injecting the cells into syngeneic BDIX rats *subcutaneously* at doses of $10^5$, $10^6$, and $10^7$ cells per animal and determining tumor volumes over a four week period. Viability of injected cells as determined by trypan blue exclusion was routinely greater than 95%. Figure 3 demonstrates the dramatic difference in growth behavior of the two cell lines despite similar growth rates in culture as described above. LP cells exhibited little if any growth subcutaneously, even at a dose of $10^7$ cells. In contrast, LN cells routinely formed large tumors in all animals. A dose-response effect was observed in the LN group, with tumors arising earlier and growing larger in animals given the highest dose of $10^7$ cells. Even when followed for over 7 weeks, rats given $10^6$ LP cells *s.c.* developed only barely detectable tumors,

**Table 1.** Expression of carbohydrate-associated antigens in LP and LN cells grown in monolayer culture and as intraperitoneal tumors

| | | *LP cells* | | *LN cells* | |
|---|---|---|---|---|---|
| *Antigen* | *MAb or Lectin* | *Monolayer* | *i.p. tumor* | *Monolayer* | *i.p. tumor* |
| Tn | 1E3 | ++ | +++ | − | ++ |
| sialyl-Tn | TKH2 | ++ | +++ | − | ++ |
| TF | PNA | − | − | − | − |
| sialyl-Le$^a$ | CA19-9 | − | − | − | − |
| sialyl-Le$^x$ | SNH-3 | − | − | − | − |
| H antigen | UEA-1 | +++ | − | +++ | − |
| MHC Class I | OX-18 | +++ | n.d. | +++ | n.d. |

n.d.—not done.

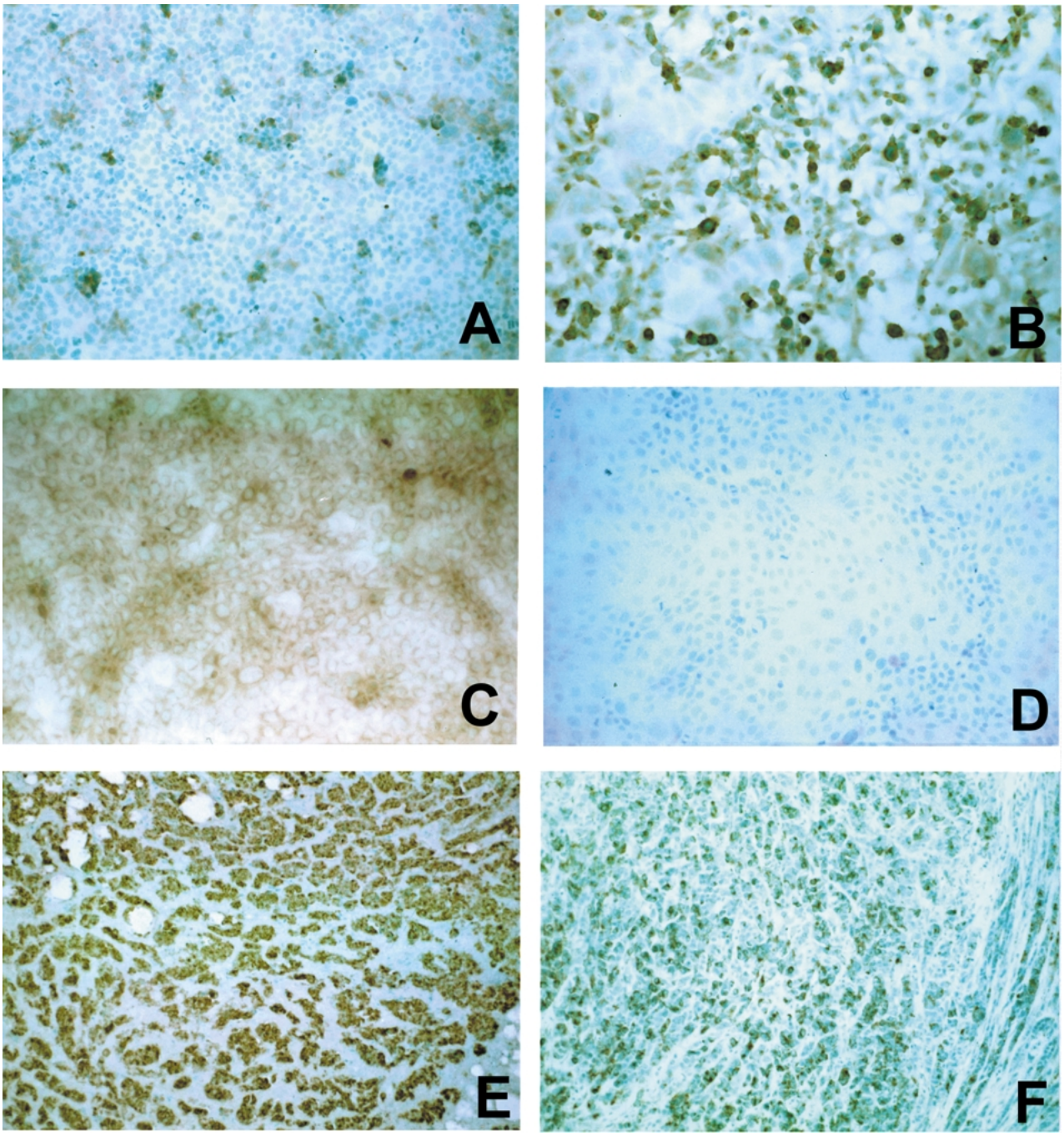


**Figure 1.** Sialyl-Tn expression in the various cell lines. In monolayer culture, K12/TRb cells (A) express STn weakly in a small population of cells, whereas the highly metastatic LMCR cell line (B) expresses STn with stronger intensity and in more cells than the parental K12/TRb cells. LP cells uniformly express STn antigen *in vitro* (C), whereas LN cells completely lack STn expression (D). When grown *in vivo* as *i.p.* tumors, LP cells maintain strong STn expression (E), and LN cells exhibit induction of STn antigen in the majority of cells (F).

whereas those given $10^6$ LN cells developed large, ulcerating tumors (data not shown).

To simulate a more physiological environment for colon cancer cells, each cell line was injected *intraperitoneally* and survival of hosts was monitored. As shown in Figure 4, at each dose tested, the animals that received LN cells had a worse survival than those receiving LP cells, revealing that LN cells are more lethal than LP cells. At autopsy, rats given LN cells *i.p.* had a higher frequency of intraperitoneal tumors, ascites, and liver metastases than rats given LP cells (Table 2).

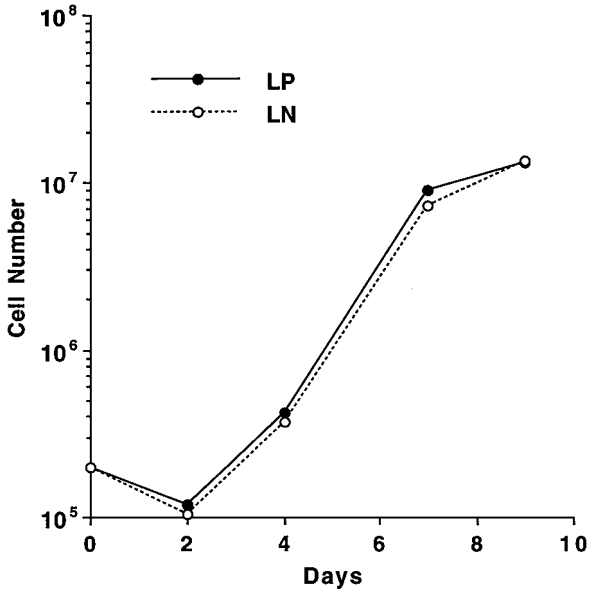


**Figure 2.** Growth rate of LN and LP cells *in vitro*. Data represent the mean of duplicate flasks.

*Carbohydrate antigen expression in vivo*

Because the LP cell line did not form tumors when injected *s.c.*, *in vivo* antigen expression could only be assessed in the few animals that developed *i.p.* tumors after high-dose inoculation of these cells. The LP tumors that formed maintained their expression of STn and Tn antigens (Figure 1E, Table 1). Curiously, however, LN cells, which lacked STn and Tn antigen expression *in vitro*, displayed a marked increase in STn and Tn antigens in *i.p.* tumors (Figure 1F). The expression of STn in LN *i.p.* tumors was typically found in approximately 50–80% of cells. Neither cell line exhibited TF, sialyl-Le$^a$, sialyl-Le$^x$ or blood group H antigen expression when grown as *i.p.* tumors (Table 1).

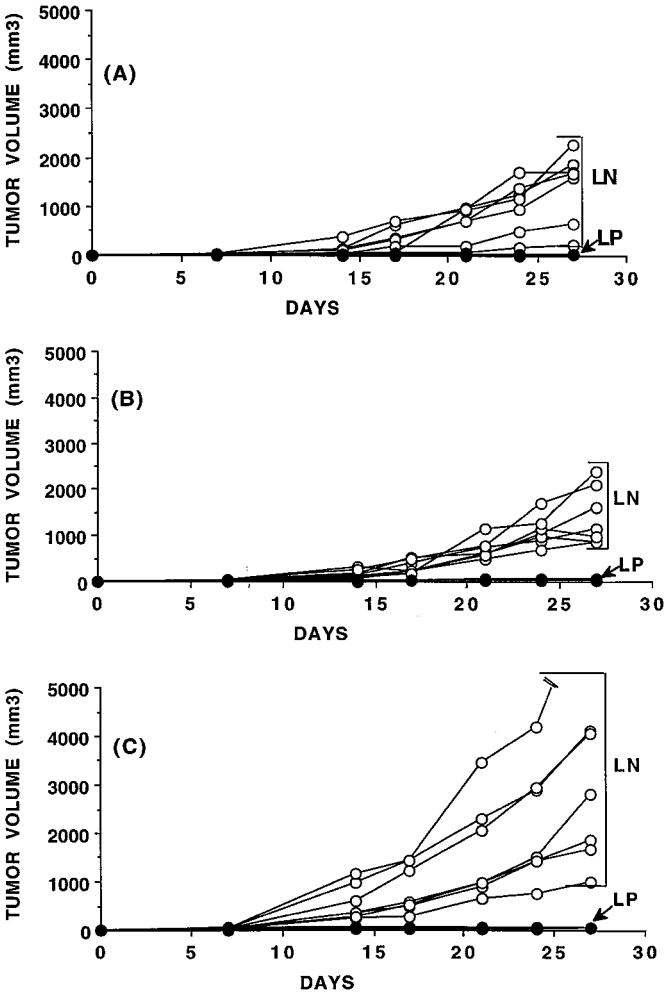


**Figure 3.** Growth rate of LN and LP cells *in vivo*. LN cells (open circles) and LP cells (closed circles) were each inoculated *s.c.* into 7 rats at doses of $10^5$ (A), $10^6$ (B), and $10^7$ (C) cells, and tumor volumes were measured. *x*-axis: tumor volume (mm$^3$); *y*-axis: days.

*Induction of STn antigen in LN tumors i.p.*

Since LN cells lacked STn expression *in vitro* but expressed the antigen *in vivo*, an experiment was performed to determine the timing of STn induction in LN tumors. Rats were challenged with $10^6$ LN cells *i.p.* and then groups of 4 rats were sacrificed on days 4, 7, 11, 14, 18, 33, and 48. The first evidence of visible *i.p.* tumors after challenge occurred on day 7, and those tumors already demonstrated STn expression in nearly half of the cells.

**Table 2.** Distribution of tumors in rats given LN or LP cells by intraperitoneal injection

| | *I.P. tumor* | | *Ascites* | | *Liver metastases* | |
|---|---|---|---|---|---|---|
| *No. cells* | *LN* | *LP* | *LN* | *LP* | *LN* | *LP* |
| $10^4$ | 3/4[a] | 0/3 | 2/4 | 0/3 | 2/4 | 0/3 |
| $10^5$ | 2/2 | 2/4 | 2/2 | 1/4 | 0/2 | 0/4 |
| $10^6$ | 3/3 | 3/4 | 2/3 | 3/4 | 1/3 | 2/4 |
| $10^7$ | 4/4 | 3/4 | 3/4 | 2/4 | 4/4 | 0/4 |
| Total: | 12/13 (92%) | 8/15 (53%) | 9/13 (69%) | 6/15 (40%) | 7/13 (54%) | 2/15 (13%) |

[a]Number of animals with tumor/number of animals innoculated.

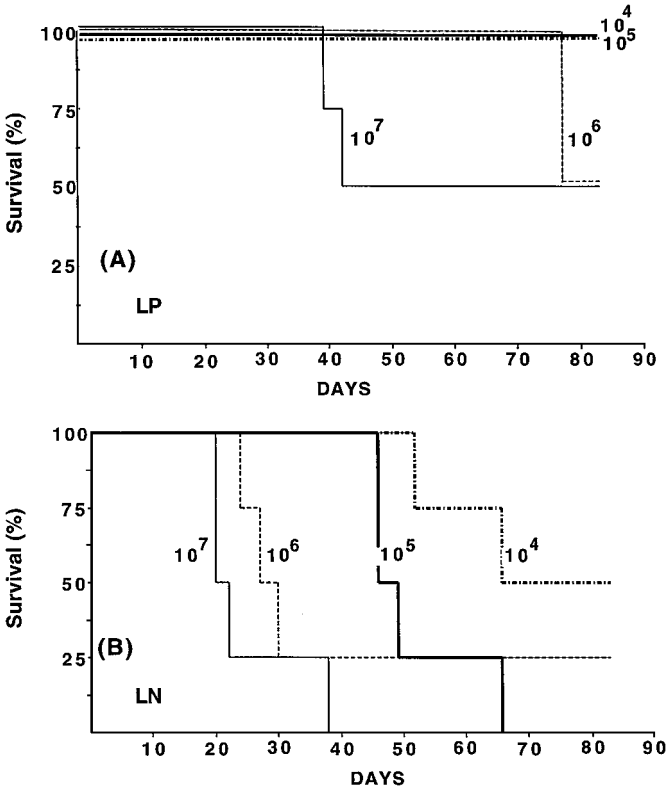


**Figure 4.** Survival of rats inoculated *i.p.* with (A) LP and (B) LN cells. Four rats were injected with each dose of cells. Results are representative of experiment repeated twice.

At all subsequent time points, all tumors detected at the time of sacrifice expressed STn; the percentage of STn-positive cells did not change in the later tumors.

*Prolonged survival by immunization with STn-KLH*

The possibility of modifying the fate of LN bearing rats was explored by pre-immunizing them with STn-KLH according to the schedule in Table 3. As shown in Figure 5, control animals immunized with KLH alone and challenged with $10^6$ LN cells *i.p.* all died of intraperitoneal carcinomatosis within 81 days of challenge. Animals that were immunized with STn-KLH but boosted with Tn-KLH (crossover group) exhibited a survival curve that was similar to controls. Likewise, the survival rate of animals given high-dose (100 $\mu$g) STn-KLH was similar to the control group. In contrast, 60% of animals immunized and boosted with low-dose (1 $\mu$g) STn-KLH were alive and well when the experiment was terminated at Day 102. At the time of sacrifice, two of the three surviving rats had no evidence of tumor, whereas one rat had a moderate amount of tumor but appeared healthy. In repeat experiments with a total of 13 animals per treatment group, rats immunized with 1 $\mu$g STn-KLH demonstrated 40–50% survival, compared to 0–20% survival for those immunized with 100 $\mu$g STn-KLH or KLH alone (data not shown).

Table 4 lists the findings at the time of autopsy or sacrifice. Tumors that arose in the KLH-immunized control group demonstrated little necrosis and a higher percentage of STn(+) cells. Likewise, animals pre-immunized with high-dose STn-KLH, or the cross-over group, exhibited minimal tumor necrosis and retained considerable STn expression in their tumors. In contrast, of the animals that were pre-immunized with low-dose STn-KLH, two never developed tumors, and in the other three, the tumors demonstrated considerable necrosis with fewer STn-positive cells.

*Immune response to STn-KLH*

To determine the humoral immune response to STn-KLH, healthy rats were immunized with either 1 $\mu$g or 100 $\mu$g of STn-KLH, and boosted twice at two-week intervals. Serum was collected after each injection and results of IgG and IgM anti-STn-KLH antibody responses are shown in Table 5. STn-KLH induced IgM and IgG responses, with antibody titers increasing after each injection. The IgG titers were higher than IgM titers.

A Winn-type assay was performed to determine whether STn-KLH-sensitized lymphocytes could affect the growth of LN tumor cells. One million LN cells were mixed with a 20-fold excess of splenic lymphocytes from animals immunized with KLH alone, STn-KLH (1 $\mu$g), or STn-KLH (100 $\mu$g) (Figure 6). As a positive control, LN tumor cells injected alone subcutaneously produced rather sizable tumors by day 25–30, and continued to grow. Lymphocytes from non-immunized animals (naïve lymphocytes) when admixed with LN cells did not affect tumor growth substantially. Likewise, lymphocytes from KLH immunized rats only delayed tumor growth to a minor extent. In contrast, when STn-KLH (100 $\mu$g) or (1 $\mu$g)-sensitized lymphocytes were admixed with LN cells, tumor growth was delayed to a greater extent.

**Table 3.** STn-KLH immunization schedule

| *Group (No. rats)* | *Immunize (Day 0)* | *Boost (Day 7)* | *Challenge*[a] *(Day 14)* |
|---|---|---|---|
| Controls (6) | KLH (1 $\mu$g) | KLH (1 $\mu$g) | LN ($10^6$ cells *i.p.*) |
| Low-dose STn-KLH (5) | STn-KLH (1 $\mu$g) | STn-KLH (1 $\mu$g) | LN ($10^6$ cells *i.p.*) |
| High-dose STn-KLH (5) | STn-KLH (100 $\mu$g) | STn-KLH (100 $\mu$g) | LN ($10^6$ cells *i.p.*) |
| Crossover group (5) | STn-KLH (1 $\mu$g) | Tn-KLH (1 $\mu$g) | LN ($10^6$ cells *i.p.*) |

[a]Pristane (1.0 ml *i.p.*) given two days before challenge.

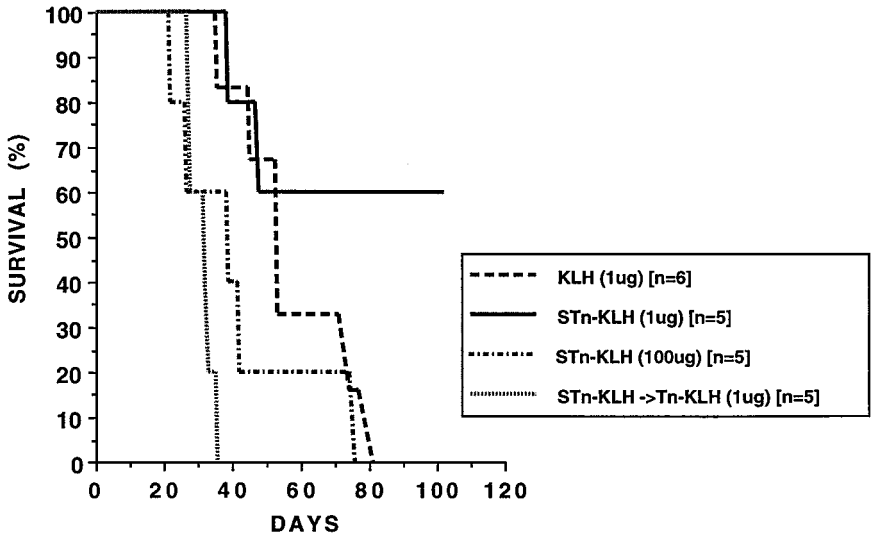


**Figure 5.** Survival of animals immunized with STn-KLH. Representative results of experiment repeated three times.

*Distribution of STn antigen expression in BDIX rats*

The distribution of STn antigen in normal organs of BDIX rats was determined by immunohistochemical staining with Mab TKH2 (Table 6). The small intestine was the only organ that expressed STn antigen. Other gastrointestinal organs, epithelial organs, and mesenchymal tissues lacked STn expression. This organ distribution of STn antigen is quite similar to that of humans.

## Discussion

The rat colon cancer cell lines TRb and TSb were originally established from a dimethylhydrazine-induced colon cancer in

**Table 4.** Tumor phenotype after immunization with STn-KLH

| | | | | *Tumor antigen expression*[b] | |
|---|---|---|---|---|---|
| *Group* | *Rat (no.)* | *Survival (Days)* | *Necrosis*[a] | *STn* | *Tn* |
| KLH | 1 | 36 | + | ++ | ++ |
| | 2 | 74 | + | ++ | + |
| | 3 | 53 | + | ++ | + |
| | 4 | 45 | + | +++ | + |
| | 5 | 81 | (na) | (na) | (na) |
| | 6 | 53 | ++ | +++ | ++ |
| STn-KLH (1 $\mu$g) | 7 | 39 | ++ | + | + |
| | 8 | 102 | (no tumor) | (no tumor) | (no tumor) |
| | 9 | 102 | (no tumor) | (no tumor) | (no tumor) |
| | 10 | 48 | +++ | ++ | + |
| | 11 | 102 | − | ++ | ++ |
| STn-KLH (100 $\mu$g) | 12 | 39 | − | +++ | ++ |
| | 13 | 42 | ++ | +++ | ++ |
| | 14 | 22 | − | + | + |
| | 15 | 27 | − | +++ | +++ |
| | 16 | 76 | + | +++ | ++ |
| STn-KLH->Tn-KLH | 17 | 33 | + | +++ | ++ |
| | 18 | 28 | + | ++ | + |
| | 19 | 28 | − | +++ | ++ |
| | 20 | 32 | + | +++ | +++ |
| | 21 | 36 | − | +++ | ++ |

[a]Degree of tumor necrosis: (−) none; (+) small; (++) moderate; (+++) large.
[b]STn or Tn positive cells: (−) ≤ 5%; (+) 6–35%; (++) 36–70%; (+++) 71–100%.

**Table 5.** Antibody response to STn-KLH

| | *IgM* | | *IgG* | |
|---|---|---|---|---|
| | *STn-KLH (1 μg)* | *STn-KLH (100 μg)* | *STn-KLH (1 μg)* | *STn-KLH (100 μg)* |
| Day 13 | 1:20 | 1:20 | 1:80 | 1:80 |
| Day 27 | 1:640 | 1:320 | 1:5,120 | 1:5,120 |
| Day 34 | 1:1,280 | 1:1,280 | 1:20,480 | 1:20,480 |

(Data from one animal but representative of experiment repeated 6 times).

BDIX rats [31]. They were selected on the basis of differential susceptibility to trypsin-mediated detachment and found to form progressive (TRb) and regressive (TSb) tumors. The progressor line has been found to express more blood group H antigen and less Tn and TF antigens compared with the regressor [28]. In those cell lines, higher expression of blood group H antigen was associated with increased resistance to NK-cell lysis whereas higher expression of Tn and TF antigens was correlated with increased sensitivity to NK-cell lysis [29].

Our group has been interested in the role of mucin and mucin-associated antigens in colon cancer biology. In our experience,

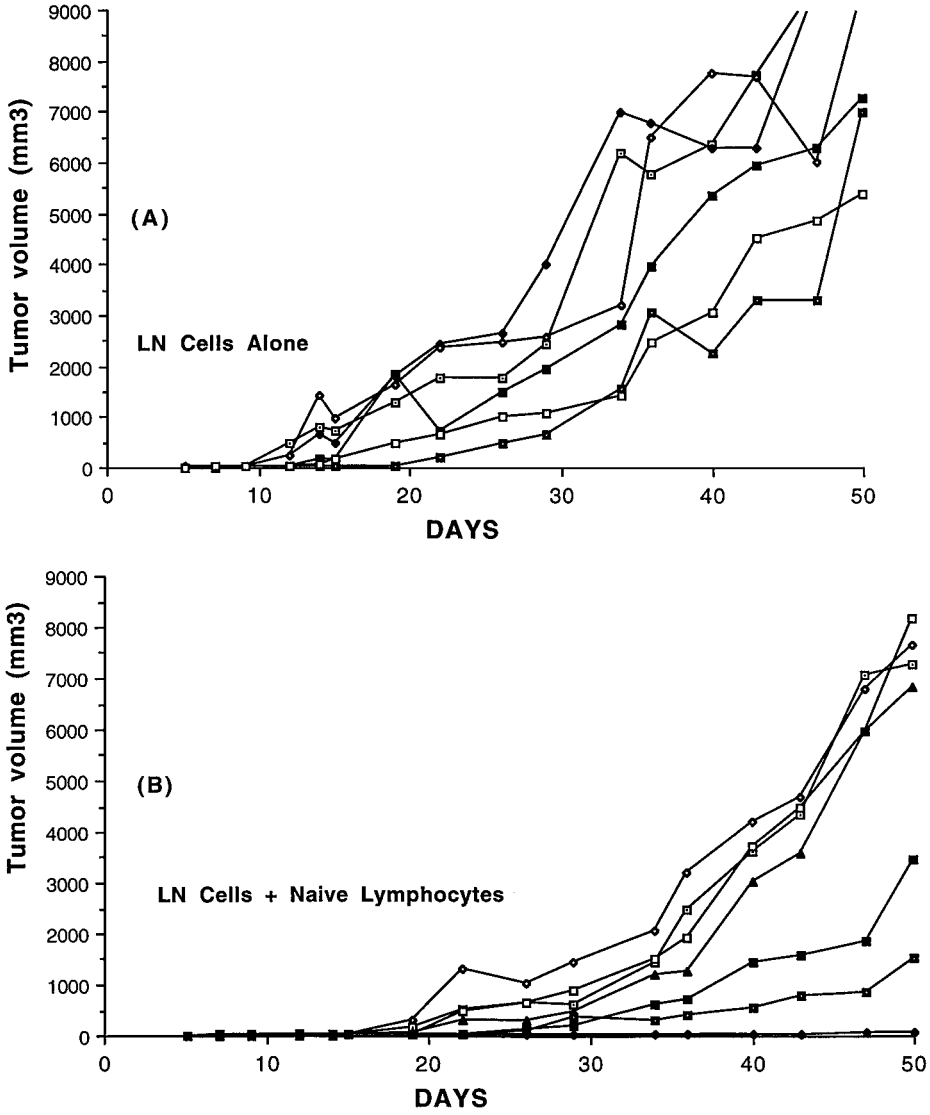


**Figure 6.** Winn-type assay. (A) Subcutaneous growth of LN cells alone, or (B) LN cells mixed with a 20-fold excess of lymphocytes from healthy (naïve) rats. Subcutaneous tumor growth of LN cells mixed with a 20-fold excess of lymphocytes from rats immunized with KLH (C), 1 μg of STn-KLH (D) or 100 μg of STn-KLH (E). *x*-axis: tumor volume ($mm^3$); *y*-axis: days after subcutaneous injection.
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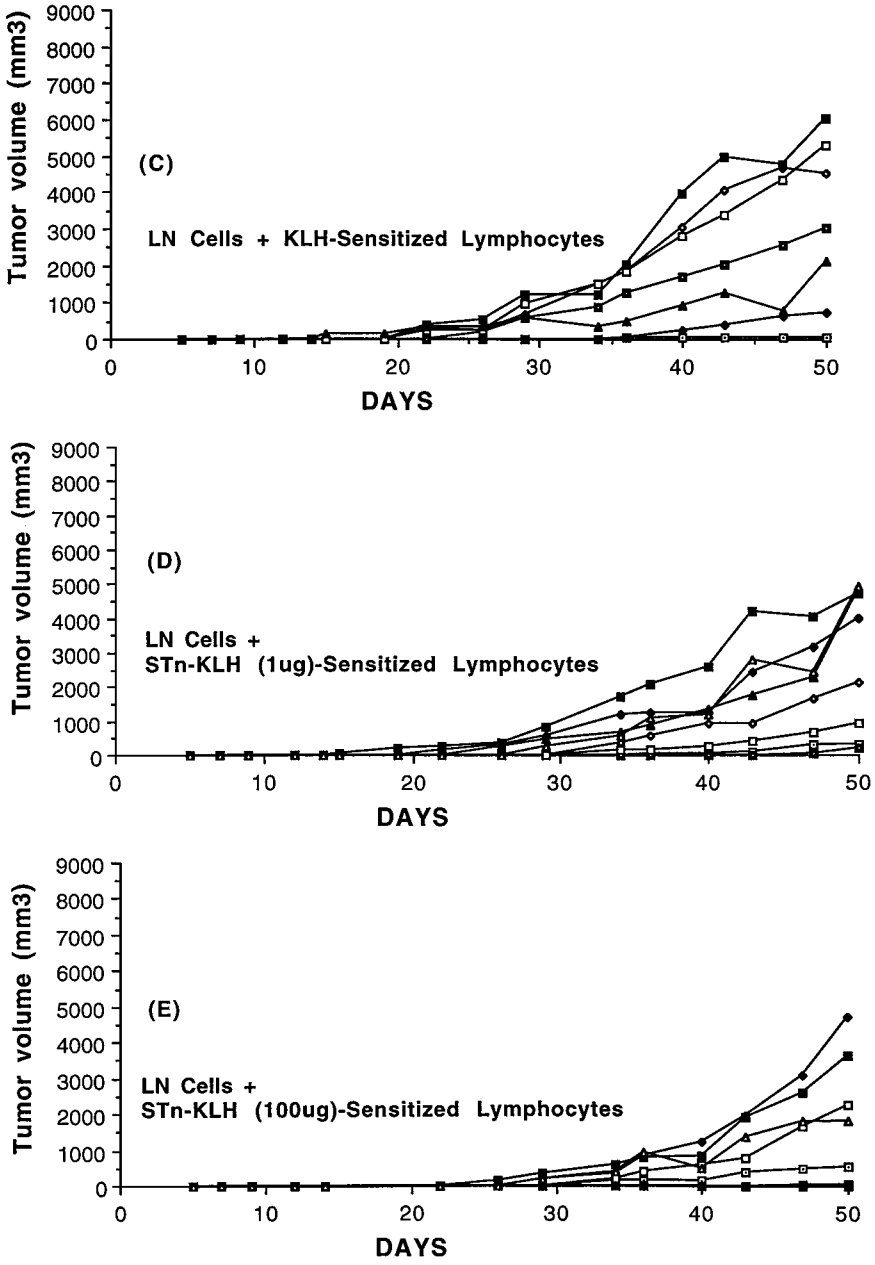


**Figure 6.** (*Continued*).

we have not detected STn antigen expression in mouse colon cancers, so we therefore sought to find an alternate animal model system. In the present study, we cloned an STn(+) and an STn(−) cell line from a derivative of rat TRb cells. We chose to use the LMCR derivative of the TRb progressor cell line because we noted that STn was expressed more strongly in this more metastatic derivative. The present investigation disclosed that the progressor phenotype co-segregated with STn(−) cells whereas regressor cells were STn-positive. This, together with the fact that these two cell lines grew at the same rate *in vitro* suggested the possibility that STn antigen may play a role in determining the fate of the cells grafted in syngeneic hosts.

The designation of LN cells as STn negative and LP cells as STn positive was based on STn expression in monolayer culture. However, when injected back into the host, both cell lines were capable of expressing STn rather strongly as *in vivo* tumors. This phenomenon is consistent with reports that TAG-72 antigen, a tumor glycoprotein that bears the STn epitope, is not expressed by cells in monolayer, but becomes expressed when cells are grown as three dimensional tumors [34]. The mechanism for the *in vivo* induction of STn antigen is not known, but the present data suggest that this may only pertain to certain antigens, since STn and Tn were induced whereas blood group H, sialyl-Le$^a$ and sialyl-Le$^x$ antigens were not. The fact that STn expression

**Table 6.** Expression of STn in normal organs of the BDIX rat

| *STn negative* | *STn positive* |
|---|---|
| esophagus | duodenum[a] (Brunner's glands negative) |
| stomach | jejunum[a] |
| colon | ileum[a] |
| pancreas | |
| liver | |
| spleen | |
| lung | |
| heart | |
| kidney | |
| bladder | |
| testis | |
| prostate | |
| adrenal | |
| brain | |
| thyroid | |

[a]Expression at crypt base; supranuclear region of cells; results representative of five animals examined.

was observed at a time when intraperitoneal tumors were just becoming grossly visible indicates that *in vivo* induction of STn antigen is an early event.

The mechanism underlying the difference in STn expression between LP and LN cells appears to be related to their expression of glycosyltransferases responsible for synthesizing O-glycan structures. LP cells have much higher $\alpha$6-sialyltransferase activity than LN cells, allowing them to synthesize more STn antigen [35]. In addition, compared to LP cells, LN cells have higher Core 2 $\beta$6-N-acetylglucosaminyltransferase activity which allows them to elongate the Core 1 structure into complex oligosaccharides, thereby diverting O-glycan synthesis away from the production of STn.

An intriguing question is why LP cells which express STn both *in vitro* and *in vivo* are more benign in host animals than LN which also express STn antigen *in vivo* (although not *in vitro*). Although we did not detect any difference in MHC class I molecule expression between the two clones, it is possible that the STn(+) cancer cells are more immunogenic, so that when they are given *de novo* to healthy animals, they can induce a brisk immune response resulting in the suppression of tumor growth. In contrast, STn(−) cells may be only weakly immunogenic allowing them to more readily establish tumor foci at which time, despite the fact that many of these cells become STn(+), the large tumor burden and possibly tumor-induced immunosuppression prevail and the animals succumb. It remains to be determined whether the two cell lines may also differ with respect to the expression of other co-stimulatory molecules or growth factor receptors to help explain their different *in vivo* behavior.

Rats immunized and boosted with STn-KLH prior to tumor challenge showed a prolonged survival compared to rats pre-immunized with KLH alone. The effect of STn-KLH was dose-dependent, such that low-dose STn-KLH was protective whereas high-dose STn-KLH was not. The greater efficacy of the low-dose over the high-dose regimen is consistent with other murine models in which low-dose immunization produces a more protective, cell-mediated immune response [36]. It is intriguing that in the tumors of rats given the more beneficial low-dose STn-KLH, there was evidence of enhanced tumor necrosis and fewer STn antigen-positive cells, suggesting an antigen-specific immune response. Further evidence of an immune response was the ability of the rats to generate IgM and IgG responses to the vaccine, and delayed tumor growth in the Winn assay.

Other evidence for anti-tumor immunity related to mucin-associated antigens has been observed in the TA3-Ha mouse mammary tumor. This highly aggressive tumor synthesizes epiglycanin, a mucin-like glycoprotein which strongly expresses the STn-related TF (Gal$\beta$1,3GalNAc-O-Ser/Thr) and Tn (GalNAc-O-Ser/Thr) epitopes [37]. Mice immunized with epiglycanin [23], synthetic TF antigen [23], or Tn antigen on desialylated ovine submaxillary mucin [24], can be protected against a tumor challenge, suggesting that a specific immune response to tumor-associated carbohydrate antigens may be therapeutic, and supporting the role of mucin in the biological behavior of tumors. In the TA3-Ha model, T cell immunity directed against the TF and Tn carbohydrate epitopes has been demonstrated [23,37,38]. The expression of truncated oligosaccharide epitopes such as Tn and TF on rat and human colon cancer cells has also been associated with an increased susceptibility to NK cell killing [29]. It is possible that STn expression on LP cells may account for their rapid elimination through a similar mechanism.

Hallouin and colleagues have also established a rat colon cancer model using clones derived from the TR rat colon cancer cell line that differed in STn antigen expression, although these clones were not specifically sorted from a parental line based on STn expression [39]. The STn-high cell line (TR2D) and STn-low cell line (PRO) demonstrated similar tumorigenicity. Upon immunization of rats with ovine submaxillary mucin as a source of STn antigen, growth of STn-high TR2D tumors was delayed, whereas growth of the STn-low PRO tumors was not affected. In our model, there were marked differences in tumorigenicity between the STn-positive and STn-negative clones. Nevertheless, immunization with synthetic STn antigen (rather than whole mucin) was able to improve survival. Taken together, these animal models suggest that vaccines based on synthetic or native STn antigens might have therapeutic benefit.

The rat colon cancer model described herein should facilitate studies on the role of STn antigen in colon cancer and the utility of STn-based immunotherapy. The LN cell line and its syngeneic host closely resemble human colon cancer in several respects. First, like LN cells, the majority of human colon cancer cell lines when grown in culture lack STn expression,

whereas 87–90% of endogenous colon cancers are STn(+) [1,13]. Second, most human colon cancers express STn heterogeneously, often with fewer than half of the cells in a tumor being STn(+). Third, like human colon cancer, LN cells are progressive, metastatic and eventually lethal to the host. Finally, the organ distribution of STn antigen in BDIX rats is quite similar to that in humans. While human colon cancer cell lines can be studied *in vivo* using immunodeficient mice, the present model affords the advantage of studying colon cancer biology in hosts with an intact immune system. In addition, the expression of STn antigen by LN cells *in vivo* permits these tumors to be localized by radiolabelled anti-STn antibodies, making these cells conducive for experiments on radioimmunodetection and radioimmunotherapy [40].

Several issues related to our findings will require further elucidation. For example, the factors responsible for inducing the *in vivo* expression of STn antigen in LN cells remain to be clarified, including whether this upregulation is at the level of glycosyltransferases versus mucin glycoprotein acceptors. Also, the fact that LN cells re-express STn antigen *in vivo* but behave more aggressively than their STn-positive counterpart LP cells, raises the question of whether STn is responsible for the aggressive behavior of cancer cells or whether this antigen represents a surrogate marker of other biological processes. In addition, the efficacy of STn-based immunotherapy for animals with established tumors remains to be further evaluated. Clinical trials of STn-KLH immunotherapy in breast cancer are underway. This model should provide the groundwork for elucidating the biological and immunological function of tumor-associated carbohydrate epitopes and may facilitate pre-clinical testing of mucin antigen-based vaccines.

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