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SHORT COMMUNICATION

A rat experimental model for the design of vaccines against tumor associated antigens Tn and Sialyl-Tn

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Clones either strongly or barely expressing the Tn and Sialyl-Tn antigens were isolated from a rat colon carcinoma cell line. Expression of the antigens in normal rat tissues was very restricted and vaccination using Ovine Submaxillary Mucin as the immunogen could delay growth of the Sialyl-Tn positive cells, but not of the Sialyl-Tn negative cells in syngeneic rats. The model should be useful for testing new anti-Tn or Sialyl-Tn vaccination protocols.

Keywords: **vaccination, Sialyl-Tn antigen, rat model**

Introduction

Aberrant glycosylation is a recurrent feature of cancer cells and as a result, carbohydrate structures, poorly expressed or hidden on normal tissues, become accessible at the surface of malignant cells. For this reason, such structures can form potential targets for immunotherapy [1]. Mucins or mucin-like proteins are major carriers of antigens such as Tn and Sialy-Tn (STn) which are short O-glycans. These correspond to GalNAcα-O-Ser/Thr and NeuAcα2-6GalNAcα-O-Ser/Thr, respectively. Vaccination protocols against the Tn antigen have been used in one animal experimental model, the TA3-Ha murine mammary carcinoma cell line of BALB/c mice [2,3]. It could be shown using this model, that antibodies can be raised against the antigen, giving a protection from tumor growth. Patients have been immunized against the STn antigen and preliminary studies indicate that patients who developed high antibodies titers in their serum survived longer than patients with lower titers [4]. The vaccine used was prepared by coupling synthetic STn epitopes to the immunogenic carrier protein Keyhole Limpet Hemocyanin (KLH) and injected with the adjuvant QS21. However, other approaches are possible, as suggested by the work of Lo-Man et al. [3] who used a multiple antigenic glycopeptide (MAG) carrying the Tn antigen associated with a CD4 helper peptide. Assaying the validity of such novel approaches and comparing the efficacy of different vaccine preparations to eliminate tumor cells *in vivo*, requires the availability of relevant animal models. However, no such animal model is available yet for the STn antigen. Despite a high *in situ* expression on many types of carcinomas including breast, colon, pancreas, ovary, stomach and lung, the STn antigen is very rarely detected on cell lines in culture [5]. In order to obtain an animal model that can allow testing of various anti-STn vaccination protocols, we screened a set of murine cell lines for their expression of the antigen and describe the isolation of a rat clonal cell line with high expression of both Tn and STn.

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Materials and Methods

Cell lines and culture conditions: The TR rat colon carcinoma cell line was a gift from Dr. F. Martin (Dijon, France). The other cancer cell lines tested were the rat lymphomas G1-TC and C58NTD, breast carcinoma MADB 106 and 13762, rhabdomyosarcomas F9-4/0, S4T18 and R9°, myeloma IR 983, fetal lung cell line RFL-6 as well as the mouse C26, CT26 and CMT93 colon carcinomas, TS/A mammary carcinoma, B16.F1 melanoma, J774 macrophage cell line, and L929 derived from adipose tissue. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, or in DMEM with the same additives. Adherent cells were sub-cultured by dispersal with 0.025% trypsin and 0.02% EDTA at 1/6 split. Cells were checked regularly for Mycoplasma contamination by fluorescent Hoescht 33258 stain.

Antibodies: Anti-Tn and anti-STn IE3 and TKH2, respectively, were kind gifts from Dr. H. Clausen (Copenhagen,

Denmark). Flow cytometry was performed as previously described [6] using FITC-labeled anti-mouse immunoglobulins from Sigma (St Louis, MO). Immunostaining was also performed as previously described [6] using the biotin/avidin antibody and 3-amino-9-ethylcarbazol (AEC) from Vector Laboratories (Burlingame, CA).

Vaccinations with Ovine submaxillary mucin (OSM): Inbred BDIX female rats were purchased from Iffa-Credo (L'Abresle, France) and housed under standard conditions in our laboratory. Two to 3 months old animals were used. Groups of 10 rats were immunized 3 times, one week apart, i.f.p. with 20 µg OSM (Accurate Chemicals, Westburry, N.Y.) emulsified in 100 µl Freund's incomplete adjuvant (Gibco BRL, Cergy Pontoise, France). Control groups received PBS emulsified in the adjuvant. One week after the last injection, animals received either 1×10^6 PRO cells or TR2D cells s.c. in the flank and tumor growth were weekly measured in the 3 dimensions with calipers. Comparison of the tumor growth in vaccinated and control animals was performed by the statistical test of "repeated-measures analysis". Serum samples from all rats were obtained at the time of the first injection (preimmune serum) and then at days 21, 28 and 35. These experiments were performed in agreement with the rules from the French Ministry of Agriculture, under supervision of the Veterinary Services (Agreement N° A44565).

Serological analysis: Serum samples from immunized and control rats were analyzed by ELISA. OSM, 1 µg/well, in distilled water was coated on ELISA plates (NUNC, Naperville, IL) by overnight incubation at 37°C. Plates were then incubated for 1 h at 37°C with 200 µl/well 3% defatted milk in PBS. Rat sera diluted at 1/50, in the same buffer, were then added and incubated overnight at 4°C. Plates were washed with PBS containing 0.1% Tween 20 before addition of a goat alkaline phosphatase-labeled anti-rat IgG (Sigma, St Louis, MO), diluted 1/10 000 in PBS containing 1% BSA and further incubation for 1 h at 37°C. After washings, reactions were revealed using p-nitrophenyl phosphate and adsorbance read at 405 nm.

Results and Discussion

A series of rat and mouse cell tumor cell lines were screened for their expression of either Tn or STn antigens by flow cytometry and immunofluorescence. None of the mouse cell lines expressed these antigens in a detectable manner. Among the rat cell lines studied, only one, TR, showed strongly positive cells for both antigens. Yet the staining was quite heterogeneous. We thus screened clones derived from this cell line that were previously obtained by Caignard et al. [7] or in our group [8]. Out of 15 clones tested, 3 showed a strong reactivity with both antibodies, 6 presented a very weak reactivity and the others were intermediate. All clones were injected s.c. to groups of syngeneic BDIX rats and their growth was monitored. Large differences in tumorigenicity

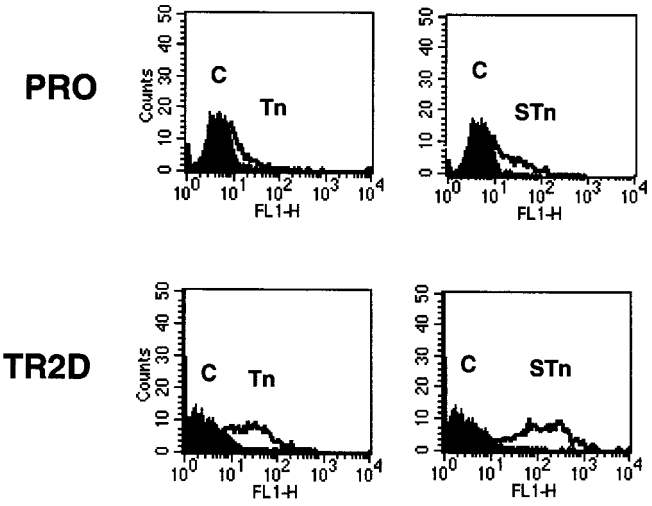

Figure 1. *In vitro* expression of Tn and STn antigens, determined by flow cytometry using IE3 and TR2D antibodies respectively, by the TR2D and PRO clones. The log of fluorescence intensities (FL-1) obtained using a FACScan flow cytometer is plotted as a function of cell number (Counts). C (black plots) represent the negative controls without primary antibodies.

were observed among clones. However, there was no relationship between tumorigenicity and Tn or STn expression (data not shown). Two clones of similar tumorigenicity were selected for further study, PRO and TR2D respectively weakly and strongly positive, as shown on Figure 1. Expression of the antigens was then tested on tumors derived from these clones in order to control that it would be maintained *in vivo*. TR2D cells were injected s.c. to syngeneic BDIX rats and the resulting tumors were excised one month later. A clear expression of the Tn and STn antigens was observed on sections from these tumors (Fig. 2 A and B).

The localization and levels of expression of carbohydrate antigens in human and murine tissues can be quite different. For example it has been shown that the ganglioside GM2 is weakly expressed in normal human tissues, but highly in normal mouse tissues. As a result, its immunogenicity in humans appears to be strong, while it is weak in mice [9]. For a tumor associated antigen to be a good target of immunotherapy, it is necessary that its expression on normal tissues be minimal and much more limited than on tumor tissue. In humans, this is the case for the Tn and STn antigens since their expression, although not negligible on a variety of epithelial tissues, is much weaker than on carcinomas. We controlled that it was also the case in rats. The binding of antibodies IE3 and TKH2 to normal rat tissues was thus determined and found to be quite restricted, especially in the case of TKH2 (Table 1). Indeed, only a very few thymic epithelial cells were strongly labeled. In addition, a weak staining of secreted material was visible in the submaxillary glands and a weak intracellular staining, probably corresponding to the Golgi apparatus was visible in the colonic epithelium. All other tissues were completely negative. Unlike in humans, no

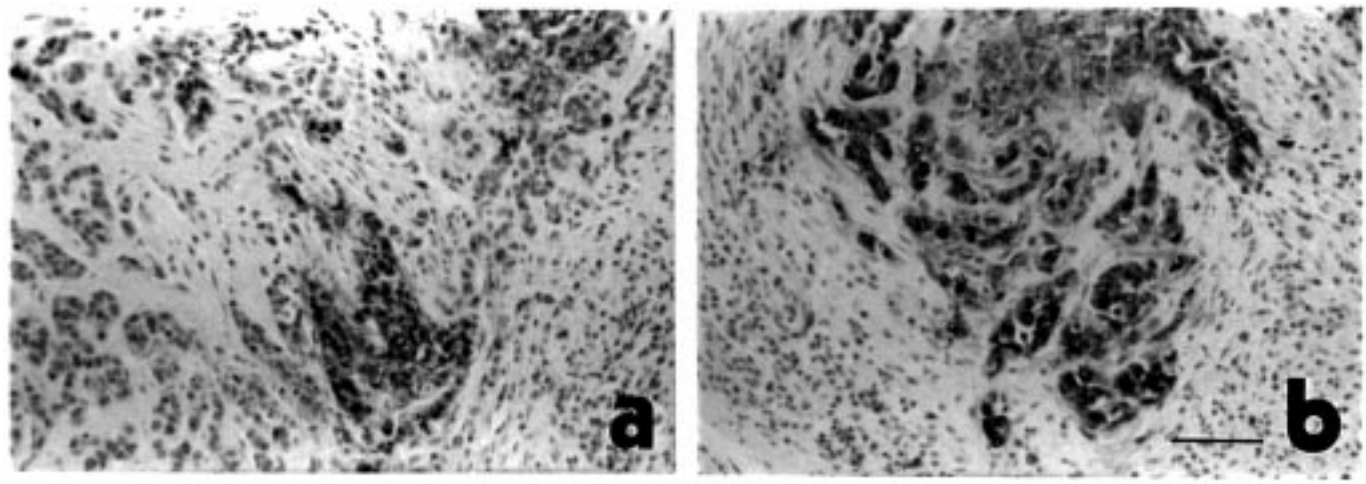

Figure 2. Expression of Tn and STn antigens, using IE3 (A) and TKH2 (B) antibodies respectively, on a TR2D tumor grown in syngeneic rats. Labeling is restricted to cancer cells and absent from the stroma. Counterstaining with hematoxylin (light gray). Bar: 100 μm

Table 1

Tissues	*Tn antigen*	*STn antigen*
Brain	−	−
Thymus	−	−/+++[a]
Heart	−	−
Lung	−	−
Salivary glands	−	+[b]
Stomach	+[c]	−
Small intestine	+[c]	−
Proximal colon	++[d]	+[c]
Distal colon	++[d]	−
Liver	−	−
Spleen	−	−
Urinary bladder	−	−
Kidney	−	−
Ovary	−	−
Skin	−	−

Expression of Tn and STn antigens in normal rat tissues. The presence of the antigens was revealed by immunohistochemistry on ethanol fixed sections of rat tissues using the IE3 and TKH2 MAbs as anti-Tn and STn respectively.
[a] Very rare, but strong labeling; [b] weak labeling of secreted material; [c] intracellular Golgi type labeling; [d] apical cellular and secreted material labelings.

staining was visible on vascular endothelium of capillaries from the digestive tract. Moreover, in humans, STn turns out to be present in the normal colonic epithelium, but is not detected by antibodies such as TKH2 because of O-acetylation of the sialic acid residue. Removal of the O-acetyl group by saponification reveals its presence [10]. Yet, treatment of rat colon sections using KOH did not reveal STn epitopes detectable by MAb TKH2 (data not shown). These results indicate that in rats, the level of expression of STn in normal tissues is even lower than in humans. The labeling given by the anti-Tn IE3 also was quite restricted, nevertheless, a significant staining could be observed in the colonic epithelium in addition to a weak intracellular Golgi-type labeling in the stomach and small intestine.

Given the very limited expression of the STn antigen on normal rat tissues and the availability of 2 cell clones, derived from the same parental tumor, with high and low expression of the antigen respectively, a vaccination experiment was conducted in order to assess the validity of the model for the design of immunization protocols. The mucin from ovine submaxillary glands (OSM) is known to carry exclusively the STn disaccharide as O-glycan. Groups of rats were thus injected with either OSM or PBS in the presence of an adjuvant and it could be shown that the rats immunized with the mucin developped anti-OSM antibodies (Fig. 3A). In order to know if this antibody response could have an anti-tumor effect, rats were challenged with either TR2D or PRO tumor cells. The growth of the STn strongly positive tumors TR2D was slowed down in animals immunized with OSM (Fig. 3B). In contrast, growth of the STn weakly positive PRO tumors was similar in animals immunized with OSM and in animals which had received only PBS and the adjuvant (Fig. 3C), indicating that the immunization against OSM provided an anti-STn immunity responsible for the tumor growth delay of a strongly STn tumor. Although the vaccination protocol using OSM an an immunogen is far from being optimal, it shows that the rat experimental model TR2D/PRO should be a good model to study the efficacy of other more sophisticated immunogens aimed at raising anti-STn or anti-Tn responses.

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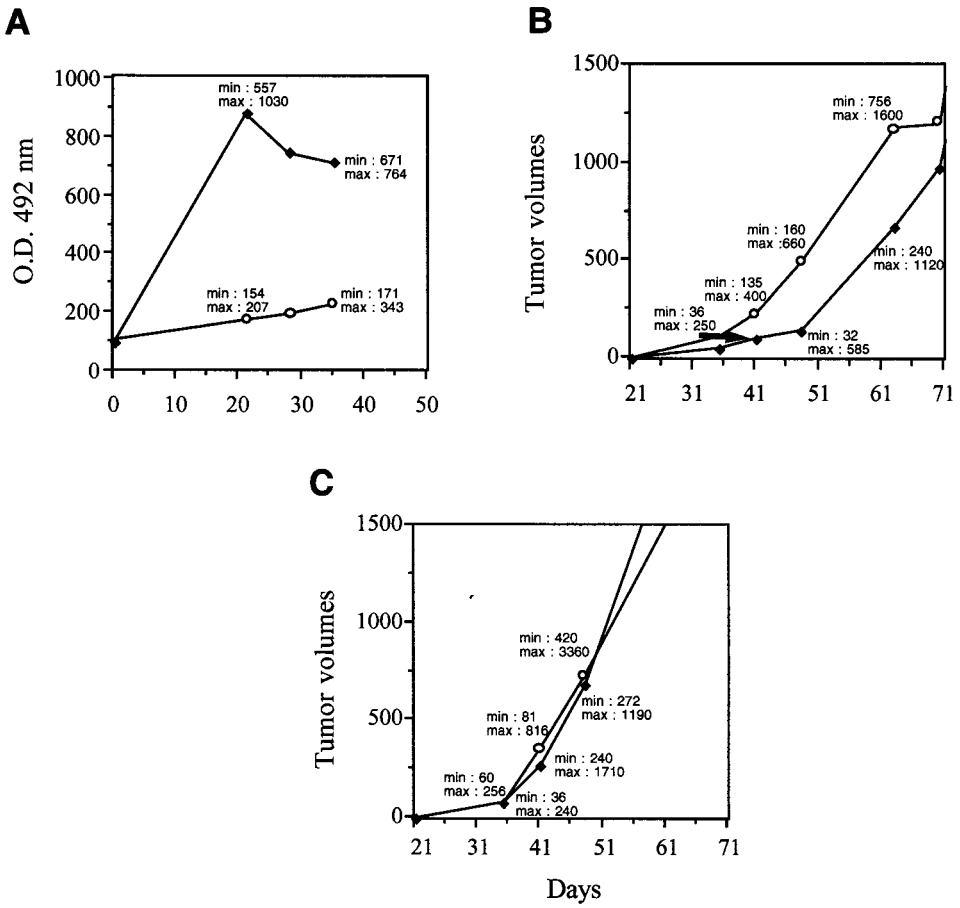

Figure 3. Mean serum reactivities against OSM and tumor volumes in rats immunized with OSM (closed symbols) or in control rats which received only PBS (open symbols). Blood was drawn at day 0, before the first injection of OSM and at days 21, 28 and 35. Reactivities were measured by ELISA using a phosphatase alkaline-labeled goat anti-mouse IgG that cross reacts with IgM. Mean values are plotted (A); after immunization, rats received s.c. 10^6 TR2D (B) or PRO cells (C). Tumor volumes were measured weekly. Mean values are plotted. Extreme values at each time point are also given. Statistical analysis by "repeated measures analysis" reveals a significant difference between the immunized and control groups in rats challenged with TR2D ($p = 0.016$), but not in rats challenged with PRO ($p = 0.468$).

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