Cancer Immunotherapy by Intratumoral Injection of α-gal Glycolipids

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Abstract. *Aim/Background: To determine the feasibility and safety of intratumoral α-gal glycolipids injection for conversion of human tumors into autologous Tumor Associated Antigens (TAA) vaccine. α-Gal glycolipids bind anti-Gal - the most abundant antibody in humans. Pre-clinical studies indicated that injected α-gal glycolipids insert into tumor cell membranes, bind anti-Gal and target tumor cells to Antigen Presenting Cells, thereby converting tumors into autologous TAA vaccines. We hypothesized that α-gal glycolipids might have similar utility in humans. Patients and Methods: Eleven patients with advanced solid tumors received one intratumoral injection of 0.1 mg, 1 mg, or 10 mg α-gal glycolipids. The primary endpoint was dose-limiting toxicity (DLT) within 4 weeks. Secondary endpoints included long-term toxicity, autoimmunity, radiological tumor response and survival. Results: There were no DLT and no clinical or laboratory evidence of autoimmunity, or any other toxicity. Few patients had an unexpectedly long survival. Conclusion: Intratumoral injection of α-gal glycolipids is feasible and safe for inducing a protective anti-tumor immune response.*

T-cell infiltration into tumors is associated with improved prognosis for several different human cancers (1-5). This infiltration implies that the human immune system is able to recognize and react against the multiple Tumor Associated Antigens (TAA) which arise in spontaneously occurring tumors. It may be possible to enhance this specific recognition and reaction and achieve immune surveillance protection against micrometastatic deposits of tumor cells elsewhere in the body which express the same TAA.

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Many TAA are unique to the tumor and absent from normal or embryonic tissues. These TAA appear as a result of genomic instability that generates multiple coding mutations specific to the malignant tissue in the individual patient. Many of these mutations result in changes in proteins, some of which may provide advantageous growth to the tumor cells (6-10). Even mutations changing individual amino acids may result in the generation of immunogenic TAA, since the immune system is capable of reacting against even very small changes in autologous proteins. Nevertheless, it is technically and logistically cumbersome – if not impossible – to identify the multiple TAA peptides in individual patients for the purpose of vaccine preparation. Therefore, the tumor itself is presently the only practical source for the full range of vaccinating autologous TAA (11).

An effective immune response against autologous TAA requires that the tumor cells and their membranes be internalized by Antigen presenting cells (APC) which then transport the TAA to the regional lymph nodes. In the lymph nodes, APC process the TAA into TAA peptides that are presented on MHC molecules for the activation of tumor-specific cytotoxic and helper T-cells (12, 13). However, tumor cells usually evolve to evade recognition by APC (6, 7, 14) and thus are "ignored" by the immune system. Therefore, uptake of vaccinating autologous tumor cells or cell membranes by APC is suboptimal as it is mediated only by random endocytosis (14, 15). As a possible solution for these problems, we have developed a novel method for *in situ* targeting of tumor cells to APC by intratumoral injection of α-gal glycolipids which then interact with the natural anti-Gal antibody (16-18).

Anti-Gal is the most abundant natural antibody in human blood, constituting ~1% of serum immunoglobulins (19). It interacts specifically with a carbohydrate antigen called "the α-gal epitope" with the structure Galα1-3Galβ1-4GlcNAc-R (20, 21). This carbohydrate epitope is produced in nonprimate mammals, but not in humans (21-23). Anti-Gal has been shown to induce rejection of pig xenografts in humans by binding to α-gal epitopes on pig cells and mediating cytolysis (24-27). We have exploited this anti-

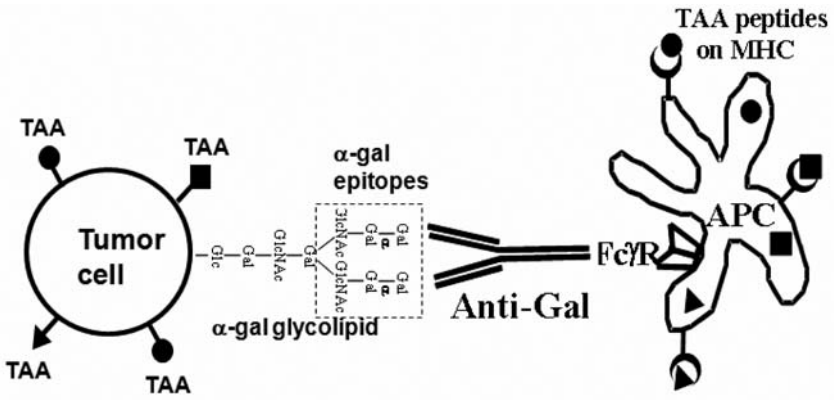

Figure 1. *In situ targeting of tumor cells to APC by intratumoral injection of α-gal glycolipids. Injected α-gal glycolipids insert spontaneously into tumor cell membranes resulting in presentation of α-gal epitopes (marked by the broken line rectangle). Binding of the natural anti-Gal IgG antibody to these epitopes is followed by interaction between the Fc portion of the bound anti-Gal and Fcγ receptors (FcγR) on the APC (illustrated as a dendritic cell). This interaction induces uptake of the tumor cells by APC and internalization of the multiple tumor-associated antigens (TAA), many of which are unique to the individual patient. The APC transport the internalized TAA to regional lymph nodes where they present the various immunogenic TAA peptides (●, ■, ▲), in association with class I and class II MHC molecules. These TAA peptides activate tumor-specific T-cells thereby eliciting a protective anti-tumor immune response.*

Gal/α-gal epitope interaction to recruit APC into tumors and target autologous TAA for effective uptake by recruited APC (17, 18). This is achieved by the use of α-gal glycolipids which are glycolipids with carbohydrate chains capped with α-gal epitopes. α-Gal glycolipids are injected into tumors in the form of micelles which spontaneously insert into tumor cell membranes *via* their fatty acid "tail". The inserted α-gal glycolipids convert injected tumors into an efficient *in situ* autologous tumor cell vaccine without requiring that TAA be identified or isolated beforehand (17, 18, 23).

Studies in the pre-clinical model of α1,3galactosyltransferase knockout (GT-KO) mouse (*i.e.* mice lacking α-gal epitopes and producing the anti-Gal antibody) indicated that binding of anti-Gal to α-gal epitopes on the injected glycolipids activates the complement cascade, resulting in the formation of complement cleavage chemotactic factors which induce effective recruitment of APC (17). Anti-Gal, bound to α-gal epitopes of glycolipids inserted into cell membranes of the tumor cells further induces their lysis by complement and by ADCC and targets these cells for effective uptake by the recruited APC (17). This uptake is mediated by binding of the Fc portion of anti-Gal IgG on tumor cells to Fcγ receptors (FcγR) on APC (Figure 1). The APC transport internalized autologous TAA to draining lymph nodes where they process and present the TAA peptides for activation of tumor-specific T-cells; ultimately eliciting a protective immune response against micrometastases expressing these TAA (17, 18).

In previous studies we demonstrated the efficacy of this α-gal glycolipids-mediated immunotherapy in the pre-clinical model of GT-KO mice bearing B16 melanoma, injected intratumorally with α-gal glycolipids (17, 18). Injections of α-gal glycolipids into a "primary" tumor produced regression of the injected tumor, prevention of tumor growth in challenged distant sites, and the arrest of established micrometastatic disease. These studies further showed that this protective immune response can be detected *in vitro* with stimulatory peptides of mouse melanoma TAA such as TRP2 and gp100. The elicited protective anti-tumor immune response was primarily dependent on $CD8^+$ T-cells and was potent enough to overcome the immunosuppressive effect of regulatory T (Treg)-cells (18).

These studies in mice have encouraged us to investigate the possibility that a similar protective anti-tumor immune response may be induced in human cancer patients by intratumoral injection of α-gal glycolipids. In this report, we demonstrate insertion of α-gal glycolipids in human tumor cells *in vitro* and summarize our findings from the first human phase 1 trial (trial registered as UM200701), aimed to determine the feasibility and safety of direct intratumoral injection of α-gal glycolipids into malignant solid tumors.

Patients and Methods

The trial was designed as a traditional phase 1 dose-escalation study. Patients were treated with a single 1.0 ml dose (0.1 mg, 1 mg, or 10 mg) of the α-gal glycolipids injected into their tumor. The primary endpoint was toxicity (CTC version 3.0) assessed over the four-week period after the injection. Based on the presumed mechanism of action for a therapeutic effect, the toxicity for this treatment, if any, was expected to be immune-related. This possibility was assessed clinically and by blood tests. Injected tumor tissue and regional lymph nodes were not available for collection and analysis in this study.

Preparation of α-gal glycolipids. α-Gal glycolipids were prepared according to a previously described method (17). One liter packed rabbit RBC (PelFreeze AK) were lysed in water. Glycolipids,

phospholipids and cholesterol were extracted from the washed RBC membranes by 20 h incubation with stirring in chloroform:methanol (1:2). α-Gal glycolipids were isolated from the extract in the aqueous phase after gradual addition of water and partition of the extract into aqueous and organic phases. The α-gal glycolipids within the aqueous phase were dried in a rotary evaporator, dissolved in water as a micelle solution and sterilized by filtration through a 0.45-μm filter. The concentration was determined by ELISA with the monoclonal anti-Gal IgM antibody M86 (17, 28). Sterility and lack of endotoxin were confirmed by standard assays.

Patients. Adult patients with malignant solid tumors which were not amenable to resection, ablation or treatment with more standard approaches utilizing chemotherapy or radiation therapy were eligible for the trial. A target lesion which could be safely injected with the α-gal glycolipids was required, as was an ECOG performance status <2 and a life expectancy ≥6 weeks. Other eligibility criteria included adequate hematological (WBC≥4500, ANC>1500, platelets ≥100,000, INR≤1.5, PTT WNL), renal (creatinine <2.2), and hepatic (Total Bilirubin ≤4) function. Patients who were being treated with other modalities had to have completed that treatment at least 2 weeks prior to entry on the trial and could not receive other treatments for six weeks after the injection. Patients with a history of autoimmune disease or patients on corticosteroids or other immunosupressants were ineligible for the study. Patients infected with HIV, Hepatitis B or C, were also excluded from the study. These human investigations were performed after approval by a local Human Investigations Committee and in accordance with an assurance filed with, and approved by the Department of Health and Human Services, (FDA IND 12946). The investigators obtained informed consent from each participant.

Analysis of α-gal glycolipids insertion. A human mammary carcinoma cell line - MCF7 was incubated for 2 h at 37˚C in constant rotation with 0.1 mg/ml or 1 mg/ml α-gal glycolipids. Subsequently, the cells were washed and incubated with the monoclonal anti-Gal IgM antibody M86 (28). After a 2-h incubation at 4˚C the cells were washed stained with FITC goat anti-mouse IgM antibody. Cells with inserted α-gal glycolipids bind M86 and this binding can be determined by flow cytometry analysis. Similar analysis was performed with human anti-Gal IgG antibody isolated from human serum, using FITC goat anti-human IgG antibody as a secondary staining antibody.

Isolation of human anti-Gal antibody. Human anti-Gal antibody was isolated from normal human serum by the use of an affinity column containing silica beads (Synsorb 115) with coupled synthetic α-gal epitopes, as previously described (21, 22). After the serum was passed through a 2-ml column, the column was washed with 40 volumes of PBS and the bound anti-Gal antibody was eluted by low pH solution of glycine-HCL (pH 2.8). The eluate was immediately neutralized and the activity of anti-Gal within the eluate was confirmed by ELISA, as described below.

Treatment. α-Gal glycolipids were injected into the targeted tumor lesion under ultrasound guidance (3 patients) or CT guidance (4 patients). Two patients had large palpable and visible tumors which were injected under manual and visual control. Two patients were injected *via* an EUS directed approach. Patients were observed closely in a Post Anesthetic Care Unit for at least an hour after injection and were admitted to the hospital for 24 h to observe for any acute allergic or complement mediated reactions. Blood tests (CBC, CMP, and DIC screen) were performed at 4 and 24 h after the injection and urinalysis was done at 24 h after the injection. Patients were evaluated at weeks 1, 2, 4, 6, 8, 12, 20-28, and at 1 and 2 years. Imaging follow-up was obtained at week 8, between weeks 20-28, at 1 year and at 2 years. Standard clinical care sometimes dictated more frequent visits, tests and imaging.

Antibody analysis. Anti-Gal: Anti-Gal IgG titer was determined as previously described (29), using synthetic α-gal epitopes linked to bovine serum albumin (α-gal BSA) as solid antigen in ELISA. Serum at serial two-fold dilutions was incubated for 2 h in ELISA wells coated with α-gal BSA. After washes, and incubation with peroxidase-coupled anti-human IgG antibody a color reaction was performed with ortho-phenylene-diamine (OPD). Patients were eligible for participating in the study if anti-Gal titer (serum dilution yielding 1 O.D.) was ≥1:50.

Autoantibodies: The patients were monitored for production of autoantibodies by ELISA. Homogenates of normal colon and skeletal muscle tissues (10 mg/ml in 50 μl aliquots of PBS) were dried overnight in ELISA wells, resulting in their firm adhesion to the ELISA wells. Wells were blocked with 1% BSA in PBS. Pre-treatment and post-treatment sera were placed in wells at serial two-fold dilutions for 2 h at room temp. Peroxidase-coupled anti-human IgM and anti-human IgG were used as secondary antibodies. The color reaction developed as in the anti-Gal assay. Production of autoantibodies was identified as an increase in antibody binding in post-treatment serum in comparison to pre-treatment serum.

Results

Baseline demographics and patients' characteristics. Eleven patients (age range 40-80 years old) with a variety of tumor histologies were treated on this phase 1 trial (Table I). The majority of the patients were male (8 patients), had metastatic cancers, and were heavily pre-treated. Most of them also had a relatively long natural history with their advanced disease prior to entry onto the trial. The three patients who did not have metastatic disease when they were injected with α-gal glycolipids, had locally-advanced pancreatic adenocarcinoma; either as recurrence after resection and adjuvant therapy (two patients) or as an unresectable tumor which had progressed after radiation and chemotherapy. Four patients (colon carcinoma, pancreatic adenocarcinoma, pancreatic neuroendocrine, renal carcinoma) had further treatment for disease progression more than six weeks after intratumoral injection.

Insertion of α-gal glycolipids into human tumor cells. In order to determine whether α-gal glycolipids, in the dose range used, display measurable insertion into human tumor cell membranes, their insertion into the cell membrane of the human mammary carcinoma cell line MCF-7 was studied. MCF-7 cells were incubated for 2 h at 37˚C in a solution containing 0.1 mg/ml and 1 mg/ml α-gal glycolipids.

Table I. *Patients' demographics and clinical characteristics (N=11).*

Characteristic	No. of patients (N=11)	%
Age, years		
Median (range)	56 (40-80)	
Gender		
Male	8	73
Female	3	27
ECOG PS		
0	8	73
1	3	27
Histology		
Pancreatic adenocarcinoma	3	27
Colon cancer	2	18
Pancreatic neuroendocrine tumor (PNET)	2	18
Prostate cancer	1	9
Renal cell cancer	1	9
Ovarian cancer	1	9
Mucinous appendiceal cancer	1	9
Stage		
Metastatic	8	73
Locally-advanced	3	27
Prior therapy	9	82
Surgery/ablation		
Chemotherapy		
0	3	27
≥2	8	73
Mode of injection		
CT/ultrasound guided	7	64
Endoscopic ultrasound (EUS)	2	18
Palpable tumor	2	18

Subsequently, the presence of α-gal epitopes on the tumor cells due to spontaneous insertion of α-gal glycolipids was determined by the binding of the monoclonal anti-Gal antibody M86 (28). The incubation with α-gal glycolipids resulted in a subsequent marked shift in both binding curves of M86 as measured by flow cytometry, indicating the presence of multiple α-gal epitopes on the tumor cells (Figure 2A). A similar antibody binding to the α-gal epitopes on the inserted glycolipids was observed with the anti-Gal IgG antibody purified from human serum (Figure 2B). These findings strongly suggest that the dose range of 0.1-10 mg/ml is likely to result also in substantial *in vivo* insertion of α-gal glycolipids into the membrane of cells within the injected tumors.

Acute toxicity and tumor response. No patient suffered either an acute complication from the procedural aspects of the intratumoral injection of α-gal glycolipids, or an immune-related reaction to that event (Table II). All patients had a completely unremarkable clinical course for the first 24 h during inpatient observation. Ten of the eleven patients had completely unremarkable clinical evaluations over the ensuing four weeks, and none of the eleven patients developed clinical signs or symptoms of an autoimmune condition during follow-up after the four week end point. One patient (#8) experienced grade 3 toxicity by week 1. He had been injected with 10 mg of α-gal glycolipids *via* an endoscopic ultrasound (EUS)-directed approach to a locally-advanced adenocarcinoma of the pancreas. The patient developed an abscess at this site requiring hospital admission, intravenous antibiotics, and drainage catheters placed by interventional radiology. With this treatment, he steadily recovered and by week 4 the patient was back to his baseline clinical state. Progression of disease was radiologically apparent by month 5 and the patient was restarted on systemic chemotherapy. After the first week, the abscess cultured out a mixed flora consistent with oral contamination of the endoscope. The protocol was then adjusted to include coverage of that maneuver with prophylactic antibiotics. No other patient experienced an infectious complication of intratumoral α-gal glycolipids injection.

All patients developed evidence of disease progression at various time points during the follow-up after the four week endpoint. However, four patients (one patient with renal cell cancer [#1], both patients with PNETs [#4 and #10] and one patient with appendix carcinoma [#11]) are alive with disease for 48+, 39+, 14+ and 13+ months, respectively, even though disease progression was evident on imaging studies (Table II). In addition, two patients with pancreatic adenocarcinoma (#7 and #8) appeared to have an unexpectedly long survival of 18 and 23 months, respectively.

Clinical laboratory tests. No clinically significant findings were found in the laboratory tests obtained around the time of the procedure or in follow-up and no patterns emerged from various abnormalities that were observed. No patients had a positive ANA at baseline, or developed one during follow-up. However, seven patients had elevations of ESR (>20 mm/hr) at baseline, and three of these patients had an ESR>90 mm/hr. Only one patient developed an increase in the ESR over the first four weeks after injection and that occurred in the patient who had developed an abscess at the injection site.

The most abnormal finding in standard blood chemistries occurred in a patient who had been heavily pretreated with systemic chemotherapy for metastatic colon cancer. This patient's total bilirubin level was 1.4 mg/dL at baseline and increased to 2.9 at week 1, and back down to 2.3 the next week and at 2.1 by weeks 4 and 6. This abnormality was associated with a rise in ALT from 24 IU/L at baseline to 51 at week 6. No other patients developed an elevation of total bilirubin or ALT. Four patients had >1+ proteinuria 24 h after injection. Two of these patients (one in 0.1 mg dose cohort and one in the 10 mg dose cohort) had a prior

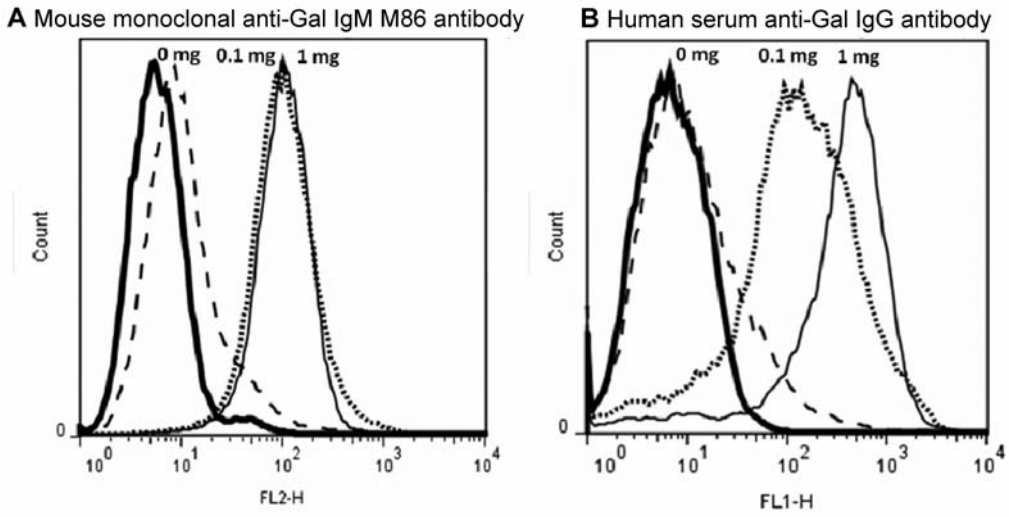

Figure 2. *Binding of the monoclonal anti-Gal M86 IgM antibody (A) and of human anti-Gal IgG antibody purified from serum (B) to human mammary carcinoma MCF-7 cells pre-incubated with 0.1 mg/ml (dotted line) and 1 mg/ml α-gal glycolipids (solid thin line), or incubated in the absence of glycolipids (dashed line). The isotype control is represented by a thick solid line curve.*

Table II. *Summary of results for the patients receiving intratumoral injection of α-gal glycolipids.*

Patient number	Tumor histology	Dose of α-gal glycolipids	Toxicity	Time-to-progression (months)	Overall survival post treatment (months)	Baseline anti-Gal titer	Autoantibody production
1	Renal cell	0.1 mg	none	42	48+ (AWD)[b]	1:80	No response[c]
2	Colon	0.1 mg	none	2	16	1:50	No response
3	Prostate	0.1 mg	none	N.D.[d]	2	1:50	No response
4	Pancreatic (NET)[a]	1.0 mg	none	24	39+ (AWD)	1:160	No response
5	Colon	1.0 mg	none	N.D.	2	1:320	No response
6	Pancreatic (adenocar.)	1.0 mg	none	2	6	1:80	No response
7	Pancreatic (adenocar.)	10 mg	none	12	18	1:80	No response
8	Pancreatic (adenocar.)	10 mg	Abscess at injected site	5	23	1:80	No response
9	Ovarian	10 mg	none	2	3	1:100	No response
10	Pancreatic (NET)	10 mg	none	12	14+ (AWD)	1:640	No response
11	Appendix (mucinous)	10 mg	none	2	13+ (AWD)	1:50	No response

[a]NET: Neuroendocrine tumor. [b](AWD): alive with disease. [c]No response: No production of autoantibodies to normal colon and skeletal muscle antigens and no ANA. [d]N.D.: Not determined.

nephrectomy and one (0.1 mg dose cohort) had advanced prostate cancer. One patient in the 10 mg dose cohort (a patient with PNET) had an increase in eosinophil count from 0.5 at baseline to a peak of 8.1 at 24 h after injection. By week 1 it was down to 4.1, and back up to 7.5 at week 6 and down again after that. Another patient in the 10 mg dose cohort had an isolated spike in eosinophil count at week 8 (2 at base line to 6.9). Although there was no clinical evidence of bleeding or thrombosis in any patient, five patients had modest (10-40) elevations of FDP in the first 24 h after injection and one of those five, as well as another patient who had normal FDP levels, also had a slightly elevated D dimer levels (0.4-0.8) 24 h after injection. However, these abnormalities did not seem to be dose-related (2 patients at 0.1 mg dose, 2 patients at 1 mg dose and 1 patient at 10 mg dose) and there were no abnormalities in platelet counts, INR or PTT.

Immunological assays for anti-Gal and autoantibody response in treated patients. This clinical trial did not enable the *in vitro* analysis of elicited immune response against the autologous TAA on tumor cells since we could not obtain tumor tissue from the treated patients. We could, however, determine in post treatment sera any changes in anti-Gal

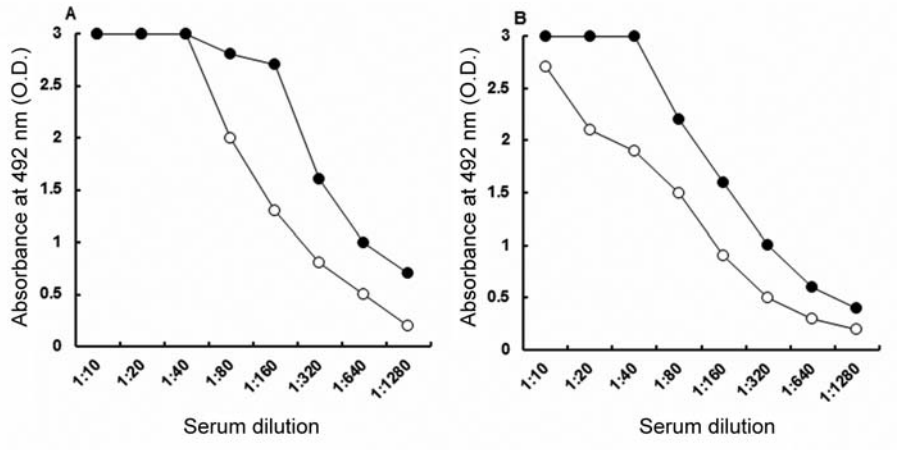

Figure 3. *Anti-Gal IgG antibody response in two representative patients treated with α-gal glycolipids. Anti-Gal response was measured by ELISA with α-gal BSA as solid phase antigen. Pre-treatment anti-Gal activity (○); Anti-Gal activity one month post treatment (●). The anti-Gal antibody titer in the two representative patients is increased post treatment by 2-4 fold.*

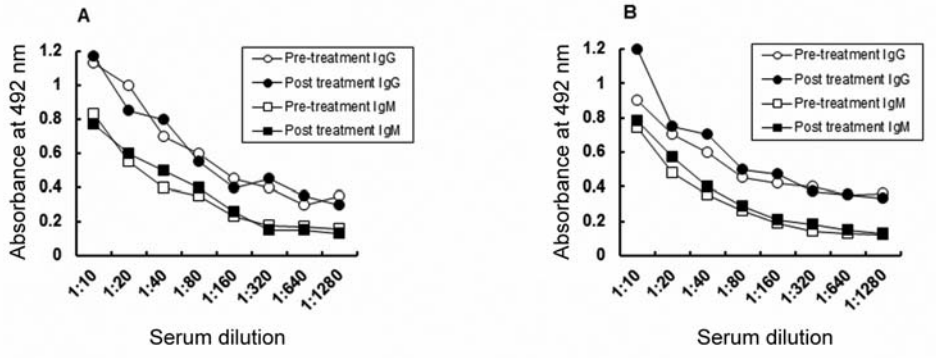

Figure 4. *Antibodies to normal colon antigens in sera of two representative colon adenocarcinoma patients treated with α-gal glycolipids. Autoantibody production was measured by ELISA with fragmented normal colon tissue (10 mg/ml) as solid phase antigen. Pre-treatment IgG antibody activity (○); IgG antibody activity two months post treatment (●). Pre-treatment IgM antibody activity (□); Post treatment IgM antibody activity (■). Both IgG and IgM antibody binding to colon normal antigens is similar in pre-treatment and post treatment sera in each patient, suggesting that no autoantibodies are produced following α-gal glycolipids treatment.*

activity as a result of intratumoral injection of α-gal glycolipids and whether the treated patients produced antibodies to normal tissue antigens. Serum anti-Gal IgG antibody activity increased by 2-4 fold after injection; indicating that α-gal epitopes introduced by the injected α-gal glycolipids activated B cells capable of producing the anti-Gal antibody (Figure 3). Nevertheless, since the activity of both IgM and IgG antibodies with normal human colon and skeletal muscle tissue homogenates (measured by ELISA) was similar in pre-treatment and post treatment sera for all eleven patients, the intratumoral injection did not appear to stimulate antibodies to normal tissue antigens (Table II). The binding curves in two representative colon adenocarcinoma patients are presented in Figure 4.

Discussion

The purpose of this clinical trial was to assess the toxicity and safety of intratumoral injection of α-gal glycolipids in humans. The only toxicity observed was an infection which appeared to be the result of the process of injection through a potentially contaminated field and which we believe was mitigated by the use of prophylactic antibiotics. There did not appear to be any toxicity related to the intratumoral injection of α-gal glycolipids *per se*. No toxicity was observed in the 24 h following the intratumoral interaction between anti-Gal and α-gal glycolipids and the ensuing complement activation due to this reaction.

A potential concern prior to this study was that effective uptake of anti-Gal opsonized tumor cells by APC might

induce an immune response to normal antigens of the tumor cells and of normal cells in the tumor. Since such normal antigens are unknown, the level of elicited antibody binding to a fragmented tissue was determined by ELISA, based on comparing IgG and IgM antibody binding in pre- and post-treatment serum samples of the individual patient. The similarity in antibody binding to normal tissue antigens in pre- and post treatment sera implies that this treatment does not cause a breakdown in immune tolerance to normal antigens. This conclusion was further supported by the lack of clinical evidence of autoimmunity.

Our previous studies on α-gal glycolipids immunotherapy in GT-KO mice show that the elicited immune response against autologous TAA is potent enough to destroy micrometastases (17, 18). Consequently, immunotherapy with α-gal glycolipids may have a particular value as neoadjuvant therapy in surgical patients with localized cancers prior to the resection of their primary tumor. Injection of the tumor 3-4 week prior to resection could allow time for the APC to internalize anti-Gal opsonized autologous tumor cells within the injected tumor, transport them to the draining lymph nodes, present the immunogenic TAA on class I and II MHC molecules and activate the immune system to react against tumor cells expressing these TAA. Thus, by the time the tumor is resected, the immune system has undergone effective activation against autologous TAA and can provide ongoing immunosurveillance against metastatic cells dispersed from the index cancer.

All patients participating in this study were in an advanced stage of their disease so it would be difficult to expect a survival benefit from such immune surveillance in this group. Nevertheless, the length of survival after injection for some of these patients seemed unusually long. This finding raises the possibility that a therapeutic effect of intratumoral α-gal glycolipid injection in a more advanced disease might be extended by combined therapy with immunomodulators such as ipilimumab (30, 31) which theoretically could nonspecifically enhance the specific T-cell response induced against autologous TAA by α-gal glycolipids injection.

We conclude that a 10 mg dose of α-gal glycolipids may be safely injected into human cancers, under antibiotic prophylaxis when injecting across a potentially contaminated field. Pre-surgical injection of *in situ* tumors may be an effective neoadjuvant strategy to elicit ongoing immune surveillance against autologous TAA after complete resection of a cancer and should be further investigated.

Conflicts of interest

The authors have no conflicts of interest and no financial interests in this study.

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