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Synthesis of α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on human tumor cells by recombinant α1,3galactosyltransferase produced in *Pichia pastoris*

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This study describes the processing of human tumor cells or cell membranes to express α-gal epitopes (Galα1-3Gal-β1-4GlcNAc-R) by the use of New World monkey (marmoset) recombinant α1,3galactosyltransferase (rα1,3GT), produced in the yeast *Pichia pastoris.* Such tumor cells and membranes may serve, in cancer patients, as autologous tumor vaccines that are targeted *in vivo* to antigen-presenting cells by the anti-Gal antibody. This rα1,3GT lacks transmembrane and cytoplasmic domains, ensuring its solubility without detergent. It is effectively produced in *P. pastoris* under constitutive expression of the P_{GAP} promoter and is secreted into the culture medium in a soluble, truncated form fused to a $(His)_6$ tag. This tag enables the simple affinity purification of rα1,3GT on a nickel-Sepharose column and elution with imidazole. The purified enzyme appears in SDS–PAGE as two bands with the size of 40 and 41 kDa and displays the same acceptor specificity as the mammalian native enzyme. rα1,3GT is very effective in synthesizing α-gal epitopes on membrane-bound carbohydrate chains and displays a specific activity of 1.2 nM membrane bound α-gal epitopes/min/mg. Incubation of very large amounts of human acute myeloid leukemia cells (1×10^9 cells) with neuraminidase, rα1,3GT, and UDP-Gal resulted in the synthesis of approximately 6×10^6 α-gal epitopes per cell. Effective synthesis of α-gal epitopes could be achieved also with as much as 2 g cell membranes prepared from the tumor of a patient with ovarian carcinoma. These data imply that rα1,3GT produced in *P. pastoris* is suitable for the synthesis of α-gal epitopes on bulk amounts of tumor cells or cell membranes required for the preparation of autologous tumor vaccines.

Key words: recombinant α1,3galactosyltransferase/*Pichia pastoris*/anti-Gal/tumor vaccines/α-gal epitope

Introduction

α1,3galactosyltrandferase (α1,3GT) is a mammalian glycosylation enzyme that catalyzes the synthesis of α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on membrane-bound and secreted glycoconjugates (Basu and Basu, 1973; Blake and Goldstein, 1981; Betteridge and Watkins, 1983; Blanken and van den Eijnden, 1985). It is active in the Golgi apparatus within the same compartment as sialyltransferases (Smith *et al.*, 1990). α1,3GT displays a unique species distribution. It is active in nonprimate mammals and New World monkeys (monkeys of South America) (Galili *et al.*, 1988). However, α1,3GT is absent in Old World monkeys (monkeys of Asia and Africa), apes, and humans (Galili *et al.*, 1988), because of the inactivation of the α1,3GT gene in ancestral Old World monkeys and apes (Joziasse *et al.*, 1989; Larsen *et al.*, 1990; Galili and Swanson, 1991). Therefore, α-gal epitopes are abundantly expressed on cells of nonprimate mammals and New World monkeys, whereas humans, apes, and Old World monkeys lack this epitope but produce against it large amounts of a natural antibody, designated anti-Gal (Galili *et al.*, 1984, 1985, 1987). The interaction between anti-Gal and α-gal epitopes is of major clinical significance, because *in vivo* binding of this antibody to α-gal epitopes on pig cells causes rejection of pig organ transplants in humans and monkeys (Good *et al.*, 1992; Galili, 1993; Collins *et al.*, 1994; Sandrin *et al.*, 1993). Anti-Gal may also be clinically exploited to enhance the immunogenicity of human autologous tumor vaccines that are modified to express α-gal epitopes (LaTemple *et al.*, 1996, 1999; Galili and LaTemple, 1997).

We hypothesized that repeated immunization with autologous tumor cells or cell membranes expressing α-gal epitopes will result in *in vivo* binding of anti-Gal IgG molecules to these epitopes (Galili and LaTemple, 1997). The bound IgG molecules target the vaccinating tumor membranes to antigen-presenting cells (APCs), such as dendritic cells and macrophages, as a result of the binding of the Fc portion of anti-Gal on the membranes to Fcγ receptors on APCs. These APCs transport the vaccinating tumor membranes to the adjacent lymph nodes, process the tumor-associated antigens (TAAs) within the tumor cell membranes, and present the TAA peptides in association with major histocompatibility complex molecules. This process, in turn, results in effective activation of anti-tumor T cells within the lymph nodes. The activated T cells can leave the lymph node, circulate in the body, and detect and destroy metastatic tumor cells expressing TAA. Recently, we demonstrated the efficacy of such tumor vaccines in protecting α1,3GT knock-out mice against the syngeneic melanoma tumor B16-BL6 (LaTemple *et al.*, 1999).

Processing human tumor cells from leukemia or lymphoma patients or tumor membranes, obtained from solid tumors, to express α-gal epitopes may be performed *in vitro* by incubation

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with recombinant (r)α1,3GT and UDP-Gal (sugar donor), according to the following reaction:

The number of exposed N-acetyllactosaminyl residues, functioning as the sugar acceptor on cell surface carbohydrate chains, may be further increased by incubation with neuraminidase, an enzyme that removes sialic acid (SA) from the terminal structure SA2-3(6)Galβ1-4GlcNAc-R (Galili and LaTemple, 1997; LaTemple *et al.*, 1996).

A major biochemical challenge in developing this novel approach for human tumor vaccine is the production of very large amounts of pure soluble rα1,3GT that will suffice for enzymatic treatment of large amounts of tumor cells ($> 1 \times 10^9$ cells) obtained from leukemia and lymphoma patients or tumor membranes (> 1g) obtained from solid tumors that are removed from cancer patients. No studies have demonstrated the synthesis of carbohydrate epitopes on such large amounts of cell membranes by recombinant glycosyltransferases. Moreover, synthesis of carbohydrate epitopes on cell membranes by full-length glycosyltransferases containing the transmembrane domain may require the use of detergents that maintain the solubility of the enzyme (Basu and Basu, 1973; Blake and Goldstein, 1981; Betteridge and Watkins, 1983; Joziasse *et al.*, 1990). Such detergents are likely to be detrimental to the integrity of the vaccinating membranes.

Previous studies have shown that solubility of glycosyltransferases may be achieved by truncation of the trans-membrane and cytoplasmic domain without affecting the catalytic activity (Colley *et al.*, 1989). For obtaining a soluble rα1,3GT, we have previously cloned a truncated cDNA corresponding to amino acids 61–376 of the New World monkey (marmoset) α1,3GT (Henion *et al.*, 1994). The produced enzyme is soluble because it lacks the trans-membrane and cytoplasmic domains. We studied the possible use of the yeast *Pichia pastoris* for production and secretion of relatively large amounts of rα1,3GT into the culture medium. Yeast culture media lack proteins and endotoxin, thus greatly decreasing the risk of such contaminants in the vaccine.

The experience in producing recombinant glycosyltransferases in yeast is very limited. The only studies describing production of mammalian glycosyltransferases in *P. pastoris* have been those of Gallet *et al.* (1998) describing production of α1,3fucosyltransferase and of Malissard *et al.* (2000), who produced soluble human β1,4galactosyltransferase, α2,6sialyltransferase and α1,3fucosyltransferase. Both studies used a methanol inducible promoter for production of the secreted enzymes. Recombinant α1,3GT could not be effectively produced in this inducible expression system. However, production of this enzyme in *P. pastoris* was found to be effective under a constitutive promoter. By using rα1,3GT produced in this system, we succeeded in synthesizing a large number of α-gal epitopes on bulk amounts of freshly obtained human acute myeloid leukemia cells and on membranes prepared from the tumor of an ovarian carcinoma patient.

Results

Constitutive expression of α1,3GT in P. pastoris

Initial attempts to produce rα1,3GT under the methanol-inducible promoter P_{AOX1} resulted in a very low yield (< 20 μg per L) (not shown). Therefore, attempts to produce the enzyme were focused on an expression system in which the P_{GAP} promoter is constitutively active in *P. pastoris* cultured in glucose-containing medium. A clone secreting high amounts of rα1,3GT was identified by measuring enzyme activity in supernatants from cultures of individual clones. This activity was determined by the synthesis of α-gal epitopes on asialofetuin in enzyme-linked immunosorbent assay (ELISA) wells (LaTemple *et al.*, 1996) and subsequent detection of the de novo synthesized epitopes by the monoclonal anti-Gal antibody M86 (Galili *et al.*, 1998). The produced enzyme which was secreted into the medium, was precipitated with 50% ammonium sulfate, dissolved in phosphate buffered saline (PBS) containing 10 mM imidazole and purified by chromatography on nickel-Sepharose column and elution with a buffer containing 250 mM imidazole.

The purity of the isolated enzymes was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). As shown in Figure 1, rα1,3GT displayed two distinct protein bands with small differences in their mobility, corresponding to 40 and 41 kD, respectively. The $(His)_6$ tag was found to be in the enzyme molecules comprising the two bands, as indicated in a western blot stained with anti-His antibody (Figure 1). These bands may result from two sizes of carbohydrate chains on rα1,3GT produced by the yeast. This enzyme has one N-glycosylation site at Asn 321 (Henion *et al.*, 1994). The presence of a carbohydrate chain linked to it is evident from its size, larger than the 37 kDa predicted from the 336-amino-acid protein chain that includes the $(His)_6$ tag. It may be possible that the lower band reflects a partial degradation of approximately 10 amino acids. As measured by the colorimetric

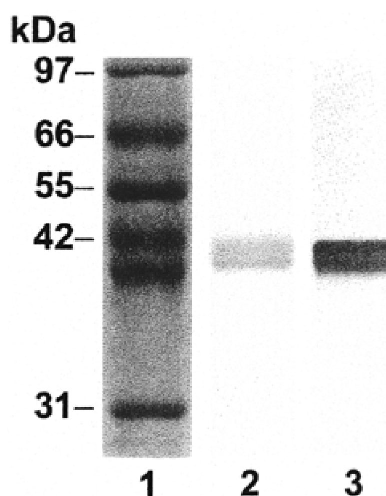

Fig. 1. Coomassie staining of SDS–PAGE (lane 2) and western blot staining with anti-His antibody (lane 3) of rα1,3GT purified from the culture medium supernatant of *Pichia pastoris* producing rα1,3GT. The two bands of rα1,3GT have a size of ~ 40 and 41 kDa. Lane 1 includes size markers.

BCA reagent assay (Pierce), 2 mg of rα1,3GT could be purified from 1 L yeast culture.

Demonstration of α-gal epitope synthesis on fetuin by rα1,3GT produced in yeast

Enzymatic activity and specificity of yeast-produced rα1,3GT was demonstrated with the serum glycoprotein fetuin, which has three N-linked carbohydrate chains with terminal structure SAα2-3Galβ1-4GlcNAc-R (Green *et al.*, 1988). The smeared bands under the main band in Figure 2A (lane 1) are likely to represent small variations in glycosylation of this glycoprotein and possibly some impurities of other serum proteins. Removal of the capping SA exposed the penultimate N-acetyllactosaminyl residues (Galβ1-4GlcNAc-R), which are the sugar acceptors for α1,3GT. Removal of SA results in faster migration of the glycoprotein in SDS–PAGE because of the smaller size of the carbohydrate chains (Figure 2A, lane 2). In the presence of the sugar donor UDP-Gal, rα1,3GT (50 μg/ml) transfers galactose to the acceptor Galβ1-4GlcNAc-R of 1 mg/ml fetuin to form the α-gal epitope (i.e., Galα1-3Galβ1-4GlcNAc-R). This elongation of the carbohydrate chain decreased the mobility of the glycoprotein in SDS–PAGE (Figure 2A, lane 3). In addition, the synthesized α-gal epitopes could be detected in a western blot both with the human natural anti-Gal (Figure 2B, lane 3) and with the monoclonal anti-Gal antibody M86 (Figure 2C, lane 3). The additional bands observed with human anti-Gal staining of lane 3 in Figure 2B may be the result of effective binding of this antibody to minute amounts of plasma proteins that contaminate the commercial preparation of fetuin and that carry α-gal epitopes as a result of the incubation with rα1,3GT.

Specific activity of rα1,3GT for synthesis of α-gal epitopes on cell membranes

As indicated above, rα1,3GT produced in yeast will be used to synthesize α-gal epitopes on vaccinating tumor cell membranes. Therefore, it was of interest to determine the specific activity of the enzyme with the Galβ1-4GlcNAc-R sugar acceptor on carbohydrate chains of membrane-bound glycoconjugates. This could not be determined by the use of trace amounts of radioactive UDP-Gal and required the use of an immunological assay that measures the full amounts of synthesized α-gal epitopes. For this purpose we used human red blood cells treated with neuraminidase to expose sugar acceptors by removal of SA. Measuring synthesized α-gal epitopes or red cells required the use of the ELISA inhibition assay, which quantifies the number of epitopes by the binding of the monoclonal anti-Gal antibody M86 in a nonradioactive assay based on the same principles as radioimmunoassays (Galili *et al.*, 1998; Stone *et al.*, 1998; Tanemura *et al.*, 2000). This assay is described in detail in *Materials and methods*. First, we determined the concentration of rα1,3GT that synthesizes the maximal number of α-gal epitopes on the red cells. The red cells were incubated at various amounts in 0.1-ml aliquots with different concentrations of rα1,3GT and assayed for binding of M86 at a high dilution (1:100) of the antibody. The extent of M86 binding was determined by removing cells and bound antibody and assessing the amount of remaining unbound M86 in ELISA with α-gal BSA (i.e., α-gal epitopes linked to bovine serum albubimn [BSA]) as solid-phase antigen. Thus, the decrease in M86 activity in the supernatant is inversely proportional to the number of α-gal epitopes per cell. The actual number of synthesized *de novo* α-gal epitopes on red cells could be determined by comparing the curve of inhibition of M86 binding to α-gal BSA to that of inhibition by rabbit red cells that express a known number of α-gal epitopes (2×10^6/cell) (Galili *et al.*, 1998).

As shown in Figure 3A, the maximal number of α-gal epitopes on 1×10^8 human red cells was synthesized by 15 μg/ml of the enzyme and corresponded to a total of 5×10^{13} epitopes. This is implied from the finding that human red cells incubated with the enzyme at this concentration expressed fourfold less α-gal epitopes than rabbit red cells, that is, the amount of treated human red cells required for 50% inhibition of M86 binding was fourfold higher than that of rabbit red cells. Because rabbit red cells express 2×10^6 α-gal epitopes per cell, the maximal number of α-gal epitopes synthesized *de novo* on neuraminidase-treated human red cells is approximately 5×10^5 epitopes per cell, that is, a total of 5×10^{13} epitopes on 1×10^8 human red cells per reaction. This number of epitopes is the maximal number of epitopes that can be synthesized on the human red cells because a threefold higher concentration of the enzyme (i.e., 50 μg/ml) yielded the same number of α-gal epitopes on the cell membranes (Figure 3A).

To determine the specific activity of rα1,3GT, we studied the kinetics of α-gal epitope synthesis on 1×10^8 human red cells in 0.1-ml aliquots containing enzyme at a concentration of 15 μg/ml, that is, the concentration of enzyme yielding maximal α-gal epitopes synthesis on the neuraminidase-treated human red cells. As shown in Figure 3B, maximal synthesis of 5×10^{13} epitopes on 1×10^8 cells was completed within 32 min. This analysis further implies that 1.5 μg enzyme per 0.1-ml aliquots synthesize an average of 1.3×10^{12} α-gal epitopes/min on the human red cell membranes (Table I). It should be stressed that the increase in the number of epitopes was not linear, probably because of differences in accessibility of N-acetyllactosaminyl acceptors on glycoconjugates of the cell membranes. Overall, these data suggest that the specific

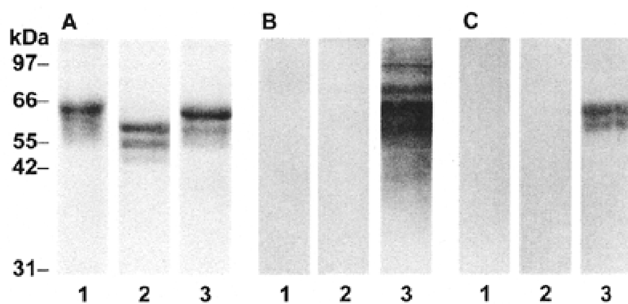

Fig. 2. Synthesis of α-gal epitopes on asialofetuin by rα1,3GT, as indicated by SDS–PAGE (**A**), western blot and staining with human natural anti-Gal IgG (**B**), or staining with the mouse monoclonal anti-Gal antibody M86 (**C**). Lane 1, original fetuin; lane 2, desialylated fetuin (asialofetuin); lane 3, α-gal fetuin produced by incubation of asialofetuin with rα1,3GT and UDP-Gal. The size of fetuin decreases on desialylation (**A**, lane 2) but increases to its original size on synthesis of α-gal epitopes on the desialylated N-linked carbohydrate chains (lane 3 in **A**, **B**, and **C**). Note that the human natural anti-Gal and mouse monoclonal anti-Gal bind only to fetuin with α-gal epitopes on its carbohydrate chains.

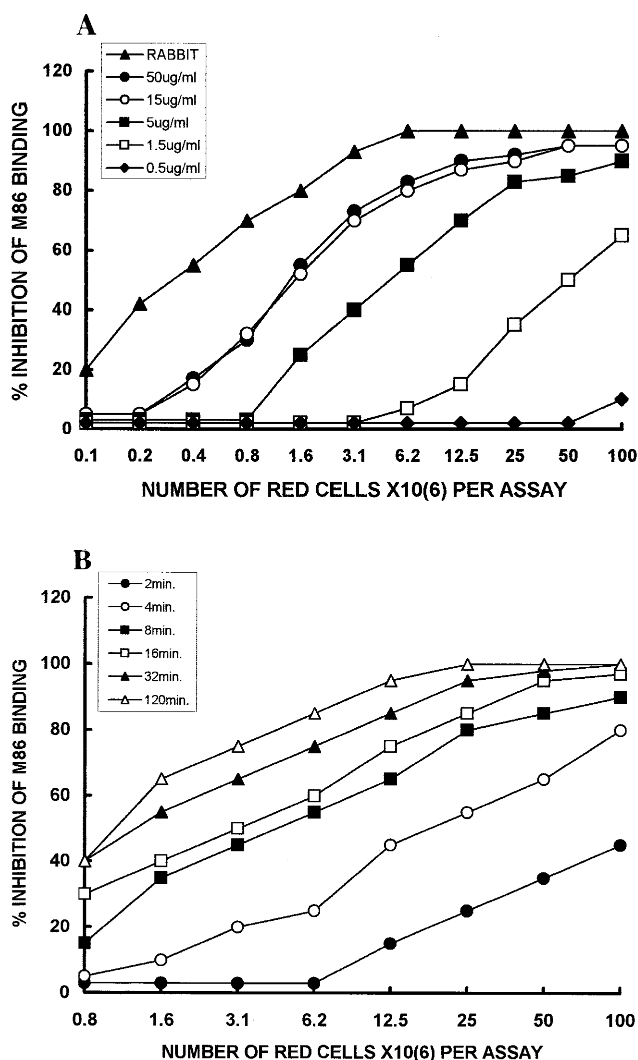

Fig. 3. Determining specific activity of rα1,3GT for the synthesis of α-gal epitopes on cell surface carbohydrate chains. (**A**) Measuring synthesis of α-gal epitopes on neuraminidase-treated human red cells at various concentrations of rα1,3GT, as determined by ELISA inhibition assays. Percent inhibition by rabbit red cells (filled triangles) or by human red cells incubated with 50 μg/ml of rα1,3GT (filled circles), 15 μg/ml (open circles), 5 μg/ml (filled squares), 1.5 μg/ml (open squares), and 0.5 μg/ml of the enzyme (filled diamonds). (**B**) ELISA inhibition assay with 1×10^8 human red cells incubated with 15 μg/ml of α1,3GT for different time periods (filled circles, 2 min; open circles, 4 min; filled squares, 8 min; open squares, 16 min; filled diamonds, 32 min). Calculation of the number of synthesized α-gal epitopes (Table I) is based on the finding in **A** that under the reaction conditions, the maximal number of α-gal epitopes synthesized on the human red cells is 5×10^5 epitopes/cell.

activity of rα1,3GT for synthesis of α-gal epitopes on membrane bound carbohydrate chains is ~ 8×10^{11} epitopes/min/μg, which corresponds to ~1.2 nM/min/mg.

Synthesis of α-gal epitopes on human leukemia cells

The treatment of leukemia and lymphoma patients with autologous tumor vaccines requires large number of vaccinating cells. It is estimated that each injection of autologous tumor vaccine should contain 5×10^8–1×10^9 vaccinating tumor cells

Table I. Number of α-gal epitopes synthesized on 1×10^8 human red cells at different incubation times[a]

Incubation time (min.)	Number of α-gal epitopes per $1{\times}10^8$ red cells
2	0.5×10^{12}
4	2.5×10^{12}
8	1.0×10^{13}
16	1.7×10^{13}
32	5.0×10^{13}
Average synthesis of α-gal epitopes per min. per 1.5 μg enzyme	1.3×10^{12}

[a]Calculations of the number of α-gal epitopes are based on the data presented in Figure 3B.

that are processed to express α-gal epitopes. Such amounts of malignant cells can be obtained in leukemia patients by leukophoresis prior to the initiation of chemotherapy treatment. The possible use of rα1,3GT produced in yeast for the synthesis of α-gal epitopes on human leukemia cells in bulk amounts of 1×10^9 cells, was studied with tumor cells of a relapsing acute myeloid leukemia (AML) patient. The cells were obtained from the patient by leukophoresis as part of his treatment.

As many as 4×10^9 leukocytes containing > 90% AML blasts were obtained from the patient. The cells, resuspended at a concentration of 1×10^8cells/ml in enzyme buffer, maintained their intactness in this solution during incubation with rα1,3GT and did not undergo lysis. Synthesis of α-gal epitopes was measured in cells incubated for 2 h at 37°C with rα1,3GT (50 μg/ml) in the presence or absence of neuraminidase (1 mU/ml). The synthesis of α-gal epitopes on the AML cells was first analyzed by ELISA with the peroxidase-conjugated lectin *Bandeiraea (Griffonia) simplicifolia* IB4 (BS lectin), which interacts specifically with α-gal epitopes (Wood *et al.*, 1979). Original AML cells and neuraminidase-treated AML cells, incubated for 2 h with UDP-Gal, washed, and dried in ELISA wells, failed to bind BS lectin (Figure 4), implying the lack of endogenous synthesis of α-gal epitopes on these cells. Cells incubated with rα1,3GT expressed α-gal epitopes as indicated by the binding of the lectin. This was the result of the presence of a certain amount of naturally uncapped N-acetyllactosaminyl residues on the leukemia cells, functioning as sugar acceptors for the enzyme. Addition of neuraminidase to the cell suspension resulted in the removal of SA from cell surface glycoconjugates and the exposure of many penultimate N-acetyllactosalminyl residues, thus increasing the number of α-gal epitopes synthesized by rα1,3GT. This was indicated by the eightfold increase in BS lectin binding to the cells (Figure 4).

Analysis of α-gal epitope synthesis on AML cells by flow cytometry

To determine whether all treated cells expressed α-gal epitopes, the binding of BS lectin was measured in a fluorescence activated cell sorter (FACS) by flow cytometry using fluorescein isothiocyanate (FITC)-labeled lectin. In accord with the data presented in Figure 4, no binding of FITC–BS lectin to untreated cells or cells treated with neuraminidase was observed by FACS analysis (Figure 5). AML cells incubated

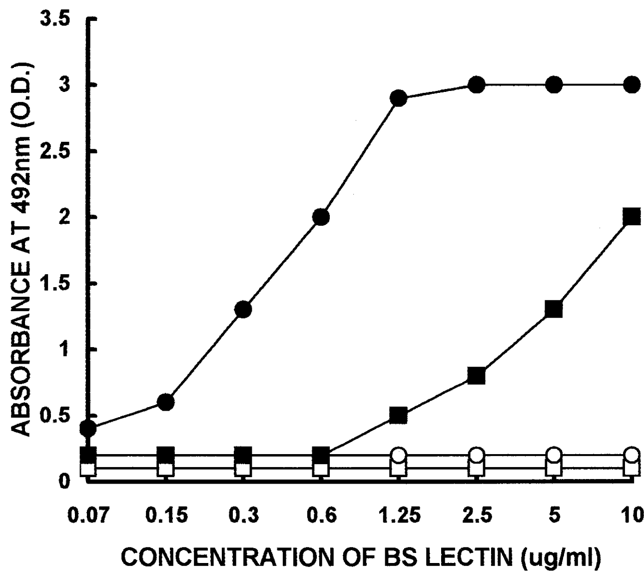

Fig. 4. Synthesis of α-gal epitopes on AML cells as measured by ELISA with HRP-conjugated BS lectin. Open squares, control AML cells; open circles, AML cells incubated with 1 mU/ml neuraminidase; filled squares, AML cells incubated with 50 µg/ml rα1,3GT; filled circles, AML cells incubated with 1 mU/ml neuraminidase and 50 µg/ml rα1,3GT.

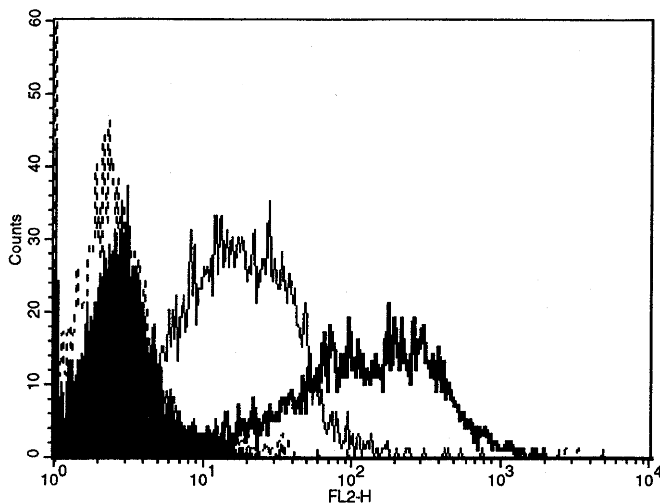

Fig. 5. Synthesis of α-gal epitopes on AML cells as measured by flow cytometry of cells stained with FITC–BS lectin. Closed histogram, untreated control cells; broken line, cells incubated only with neuraminidase; thin solid line, cells incubated only with rα1,3GT; thick solid line, cells incubated with both neuraminidase and rα1,3GT.

with rα1,3GT displayed binding of the lectin, however: incubation of the cells with neuraminidase and rα1,3GT resulted in a fivefold increase in lectin binding. As shown in Figure 5, there is no overlap between the histogram of BS lectin binding to AML cells treated with neuraminidase and rα1,3GT and that of lectin binding to untreated cells. This implies that 100% of the cells treated with the two enzymes expressed α-gal epitopes therefore bound the lectin.

Quantification of synthesized α-gal epitopes on AML cells

The treated AML cells were subjected to ELISA inhibition assay for determining the number of α-gal epitopes synthesized per cell (Figure 6). As expected, control AML cells or cells treated with neuraminidase did not bind the monoclonal anti-Gal antibody M86 even at a concentration of 40×10^6 cells/ml. The cells that were incubated with rα1,3GT in the absence of neuraminidase were 16-fold less effective in binding M86 in comparison to rabbit red cells. Because rabbit red cells express 2×10^6 epitopes/cell, these treated leukemia cells express ~ 1.4×10^5 epitopes/cell. In contrast, AML cells incubated with both neuraminidase and rα1,3GT were threefold more effective than rabbit red cells in binding M86, implying that the number of α-gal epitopes synthesized *de novo* on the leukemia cells was ~ 6×10^6 epitopes/cell.

Identification of AML glycoproteins with α-gal epitopes

The treated AML cells were further subjected to western blot analysis to identify the membrane-bound glycoproteins on which α-gal epitopes are synthesized by rα1,3GT. Each of the cell preparations were loaded as 20 µg membranes per lane in SDS–PAGE, and the blotted glycoproteins were immunostained with 10 µg/ml of human anti-Gal isolated from blood type AB serum (Galili *et al.*, 1987, 1988). As shown in Figure 7, no anti-Gal binding was observed in lanes 1 and 2 containing glycoproteins of membranes from untreated cells or cells treated with neuraminidase, respectively. Similarly, no staining was observed with glycoproteins of membranes from

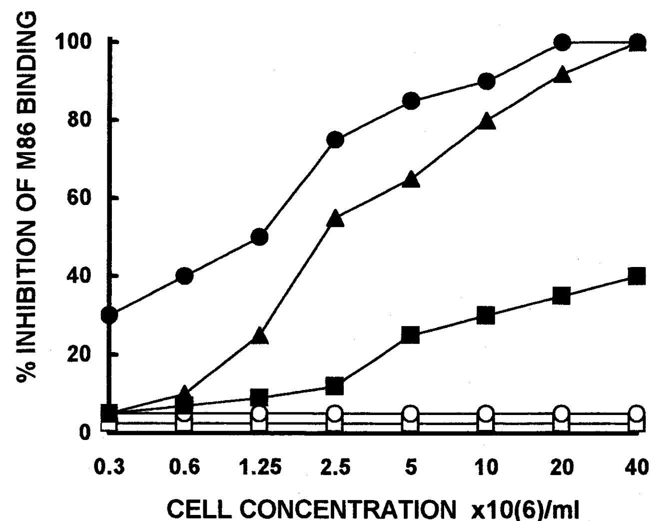

Fig. 6. Quantification of α-gal epitope expression on AML cells as measured in the ELISA inhibition assay. Open squares, control AML cells; open circles, AML cells incubated with 1 mU/ml neuraminidase; filled squares, AML cells incubated with 50 µg/ml rα1,3GT; filled circle, AML cells incubated with 1 mU/ml neuraminidase and 50 µg/ml rα1,3GT. Rabbit red cells served as standard control, which express 2×10^6 α-gal epitopes/cell (filled triangles).

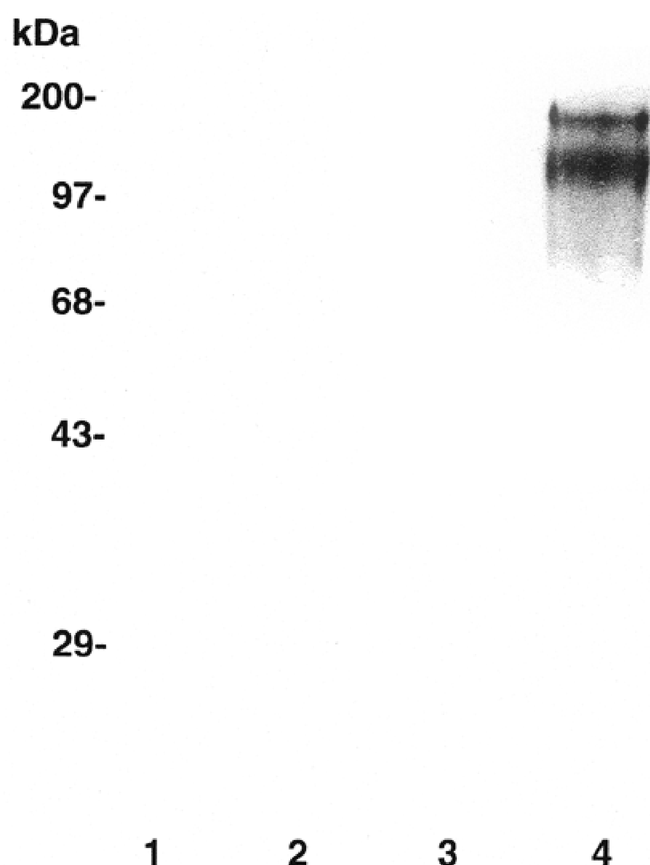

Fig. 7. Western blot staining of AML cells with the human natural anti-Gal antibody. Lane 1, control AML cells tumor; lane 2, AML cells tumor membranes incubated only with neuraminidase; lane 3, AML cells tumor membranes incubated only with rα1,3GT; lane 4, AML cells incubated with both neuraminidase and rα1,3GT.

cells treated only with rα1,3GT (lane 3), probably because the number of α-gal epitopes on these membranes was too low to be detected in this assay. However, glycoproteins of membranes from cells treated with neuraminidase and rα1,3GT were stained by human anti-Gal (lane 4). The size of most of the stained glycoproteins was within the range of 90–200 kDa. These findings imply that α-gal epitopes are synthesized primarily on membrane glycoproteins of relatively large size, possibly because the carbohydrate chains on these glycoproteins are more accessible to rα1,3GT than those on smaller glycoproteins. It is also possible that the number of carbohydrate chains on smaller glycoproteins may be too low for detection of α-gal epitopes by immunostaining of the western blots with anti-Gal.

Synthesis of α-gal epitopes on tumor membranes from human ovarian carcinoma

Preparation of autologous tumor vaccines from solid tumors differs from that in leukemias and lymphomas. This is because it is difficult to obtain large numbers of intact tumor cells from solid tumors, such as ovarian carcinoma or mammary carcinoma. We have previously suggested that solid tumors removed from patients may be used for preparation of autologous tumor vaccines by homogenization of the tumor mass and processing of the washed tumor membranes to express α-gal epitopes (Galili and LaTemple, 1997). It is estimated that at least 1 g of processed membranes is needed for vaccination of patients with the autologous tumor. To study the possible preparation of bulk amounts of tumor membranes for this purpose, we have used membranes from the tumor of a patient with ovarian carcinoma. Tumor membrane suspension of 200 mg/ml was incubated for 2 h at 37°C in the enzyme buffer containing UDP-Gal, neuraminidase, and rα1,3GT, as above. In parallel, membranes were incubated only with neuraminidase or rα1,3GT. Each membrane suspension was at a volume of 10 ml. At the end of incubation the membranes were washed, dried in ELISA wells at 100 μg/well, and assayed for expression of α-gal epitopes by measuring binding of BS lectin.

α-gal epitopes were readily synthesized on membranes incubated for 2 h only with rα1,3GT (Figure 8). However, the number of these epitopes doubled on membranes incubated with rα1,3GT and neuraminidase. No α-gal epitopes were detectable on tumor membranes treated only with neuraminidase or on untreated membranes. Because the difference in BS lectin binding to membranes treated only with rα1,3GT and to membranes treated with both neuraminidase and rα1,3GT is smaller than that observed in AML cells, it is probable that the proportion of naturally uncapped N-acetyllactosaminyl residues on ovarian carcinoma membranes is higher than that on AML cells. Nevertheless, in both tumors, addition of neuraminidase results in a marked increase in synthesis of α-gal epitopes because of the increase in the number of sugar acceptors for rα1,3GT.

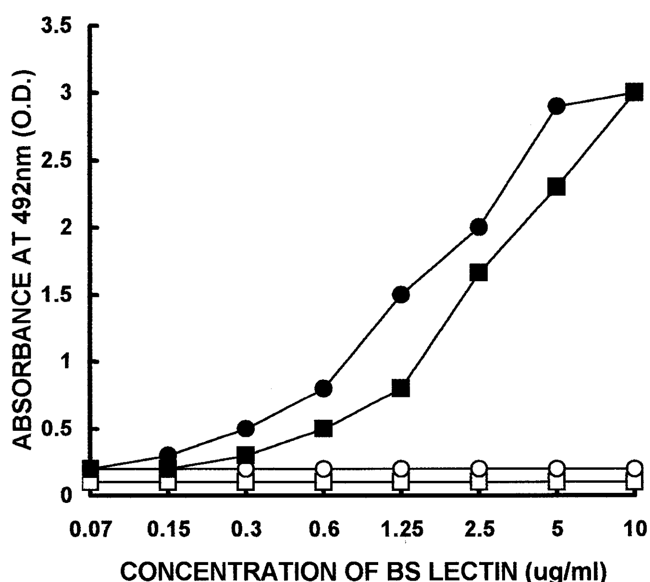

Fig. 8. Synthesis of α-gal epitopes on ovarian carcinoma tumor membranes as measured by ELISA with HRP-conjugated BS lectin. Open squares, control tumor membranes; open circles, tumor membranes incubated with 1 mU/ml neuraminidase; filled squares, tumor membranes incubated with 50 μg/ml rα1,3GT; filled circles, tumor membranes incubated with 1 mU/ml neuraminidase and 50 μg/ml rα1,3GT.

Discussion

This study demonstrates for the first time the use of a recombinant glycosyltransferase in synthesizing a carbohydrate epitope on large amount of human tumor cells or cell membranes. By using rα1,3GT produced in yeast and neuraminidase, SA on SA-Galβ1-4GlcNAc-R carbohydrate chains was replaced with galactose, to form as many as 6×10^6 Galα1-3Galβ1-4GlcNAc-R (i.e., α-gal epitopes) per myeloid leukemia cell on a total of 1×10^9 cells in enzyme reactions containing 1×10^8 cells/ml. These epitopes readily bound BS lectin, the mouse monoclonal anti-Gal antibody M86, and the human natural anti-Gal IgG. Similarly, α-gal epitopes could be synthesized on as much as 2 g of human ovarian carcinoma membranes, obtained from a tumor mass removed from a patient. These findings imply that future preparation of autologous tumor vaccines by expression of α-gal epitopes on bulk amounts of tumor cells or cell membranes will be feasible with rα1,3GT produced in *P. pastoris*. The recombinant enzyme originally cloned from a New World monkey (Henion *et al.*, 1994) lacks the transmembrane and cytoplasmic domains. Therefore, it does not form aggregates in the absence of detergent, a characteristic required to prevent alteration of the vaccinating membranes by detergents. The need for such detergents for the solubilization of the full-length rα1,3GT was previously demonstrated by Joziasse *et al.* (1990) in preparations of bovine rα1,3GT produced by the baculovirus expression system within inclusion bodies of the mosquito sF9 cell line

Previous attempts to produce large amounts of truncated marmoset rα1,3GT in other expression systems proved to be inadequate for preparation of human tumor vaccines. Most (> 97%) of the rα1,3GT produced in bacteria was found to reside within inclusion bodies in an insoluble form (Galili and Anaraki, 1995). Attempts to solubilize the recombinant enzyme within the inclusion bodies (e.g., by guanidinium chloride) resulted in its inactivation. In addition to the relatively low amounts of soluble rα1,3GT isolated from bacterial lysates, the possible contamination with bacterial endotoxin makes this enzyme unsuitable for tumor vaccines. The enzyme was subsequently produced in the mosquito cell line sF9 infected with baculovirus containing the α1,3GT gene (Henion *et al.*, 1997). Because the enzyme in this system is isolated from a cell lysate, there is the concern that the treated patient may develop hypersensitivity/allergic reaction to minute amounts of mosquito proteins that are not detectable by standard analytical methods and that may adhere to the vaccinating tumor membranes. In contrast, the medium used for yeast growth, the source for the secreted enzyme, contains only small peptides but no proteins. Therefore, the enzyme tagged with $(His)_6$ that is isolated from yeast culture supernatant on nickel-Sepharose column is free of contaminating proteins. Moreover, because the rα1,3GT produced in *P. pastoris* does not involve lysis of cells or bacteria, its purification is much easier than the previously reported isolation from bacterial or cell lysates (Galili and Anaraki, 1995; Henion *et al.*, 1997).

Recombinant α1,3GT was not effectively produced in *P. pastoris* under a methanol-inducible promoter P_{AOX} (not shown). This was the result of low expression and partial inactivation of the enzyme, which may be caused by the methanol in the medium. In contrast, the enzyme was effectively produced in *P. pastoris* under constitutive expression of the P_{GAP} promoter, resulting in the isolation of 2 mg rα1,3GT per L culture. This amount is similar to that reported for several other recombinant proteins produced in *P. pastoris* (Guo *et al.*, 1995; Weiss *et al.*, 1995). The enzyme could not be isolated directly from the culture medium, possibly because of histidine-containing peptides within the medium, which compete with the $(His)_6$ tag of the enzyme for binding to the nickel-Sepharose column. However, after the enzyme was precipitated by high salt concentration and then dissolved in PBS containing 10 mM imidazole, it readily bound to the nickel-Sepharose column. The enzyme eluted by 250 mM imidazole from these columns was found to be very pure, as indicated by the lack of other bands in SDS–PAGE gels. Analysis of the K_m of this enzyme (not shown) was found to be similar to that of the same enzyme we have previously produced in mosquito sF9 cells (Henion *et al.*, 1997). The isolated enzyme was found to be highly stable at 4°C and in a frozen form, in the presence of glycerol as stabilizer. However, repeated thawing and freezing resulted in loss of activity (not shown).

The rα1,3GT produced by yeast is most suitable for synthesizing α-gal epitopes on carbohydrate chains of tumor cells or cell membranes. Our data imply that incubation of as much as 200 mg/ml tumor membranes or 1×10^8 cells/ml with 1 mU/ml neuraminidase, 50 μg/ml rα1,3GT, and 1 mM UDP-Gal for 2 h at 37°C, represents optimal conditions for producing the maximal number of α-gal epitopes on the membranes. The recombinant enzyme is effectively removed from the treated membranes by the repeated washes and the use of 1 mM ethylenediaminetetraacetic acid (EDTA) in the washing buffer. Thus, autologous tumor vaccines that will be used in humans are likely to contain only the autologous membranes expressing α-gal epitopes and no contaminants.

This method of α-gal epitope expression on vaccinating tumor membranes can be used with any type of human tumor membranes, because all human cells have carbohydrate chains with the terminal structure SA-Galβ1-4GlcNAc-R. Future studies on the vaccination of cancer patients with autologous tumor vaccines expressing α-gal epitopes will determine whether the autologous tumor vaccines effectively induce stimulation of the immune system in humans, achieved by anti-Gal-mediated targeting of such vaccines to APCs. It may be possible that in some of the patients, the immune response to such vaccines will be effective enough to induce immune-mediated elimination of residual tumor cells that survive chemotherapy and irradiation treatments, and thus prevent the relapse of the disease.

Materials and methods

Reagents

The expression vector pGAPZαA and *P. pastoris* strain GS115 were purchased from Invitrogen (San Diego, CA). Restriction enzymes and other enzymes used for molecular cloning were from New England BioLabs (Beverly, MA). UDP-Gal was received as a generous gift from Neose Pharmaceuticals (Horsham, PA). Neuraminidase from *Vibrio cholera* and fetuin from bovine fetal serum were purchased from Sigma (St. Louis, MO). The monoclonal anti-Gal antibody designated M86 was obtained in tissue culture supernatants of the hybridoma M86 cells, as previously described (Galili *et al.*, 1998).

BSA with linked synthetic α-gal epitopes (designated α-gal BSA) was purchased from Dextra Inc. (Reading UK). The natural human anti-Gal was isolated from human AB sera by affinity chromatography on columns with synthetic α-gal epitopes (Chembiomed, Edmonton, Canada), as previously described (Galili *et al.*, 1985, 1987). Horseradish peroxidase (HRP)–conjugated anti-human IgG was purchased from Dako (Denmark), HRP-anti-mouse IgM and HRP-anti-mouse IgG from Axcell (Westbury, NY), and anti-His from Invitrogen. HRP and FITC-conjugated BS lectin were purchased from Vector Laboratories (Burlingame, CA).

Medium

The medium used was YPD, including the following materials: 1% yeast exract (Fisher Biotech, NJ), 2% peptone (Beckton Dickinson, MD), and 2% glucose (Sigma).

Tumor cells and cell membranes

Four billion leukemia cells were received from an AML patient in relapse who was treated for the removal of the tumor cells by leukophoresis. An ovarian carcinoma tissue (~ 10 g) was received from a patient undergoing surgery for the removal of the tumor. Both patients were treated at Rush Presbyterian–St. Luke Medical Center (Chicago, IL), and the tumor materials were obtained under informed consent.

Construction of vectors for expression of α1,3GT in P. pastoris

The truncated portion of the New World monkey (marmoset) α1,3GT cDNA (Henion *et al.*, 1994) containing the catalytic domain and part of the stem region but lacking the trans-membrane and cytoplasmic domains, was inserted into the expression vector pGAPZαA. For this purpose, the region coding for amino acids 61–376 in the cDNA was amplified by polymerase chain reaction (PCR) with addition of *Bam*HI site at the 5′-end and *Hind*III site at the 3′-end. The resulting PCR products were digested with *Bam*HI and *Hind*III and cloned into the pQE-32 vector (Qiagen) at the *Bam*HI and *Hind*III sites. The cloned α1,3GT gene sequence was found to be in frame at the 5′-end with the upstream $(His)_6$ coding sequence. Digestion of the resulting vector with *Eco*RI and *Hind*III produced a DNA fragment containing α1,3GT gene and its upstream $(His)_6$ tag coding sequence. This $(His)_6$ tag attached to the enzyme is needed for affinity isolation of the enzyme by nickel-Sepharose columns. Subsequently, the *Hind*III site was blunted by filled-in reaction with Klenow fragment of DNA polymerase. This *Eco*RI–*Hind*III^{klenow} fragment was subcloned into *Eco*RI and *Not*I^{klenow} sites of pGAPZαA. This vector containing a zeocin resistance gene and α1,3GT cDNA was linearized with AvrII and transformed into the yeast *P. pastoris* strain GS115 by electroporation, according to the manufacture's protocol. The transformants with stable integration of the α1,3GT gene were selected in presence of 1 mg/ml zeocin.

Isolation of P. pastoris clones producing α1,3GT

Fifty individual clones grown in presence of zeocin were screened for production of the enzyme. The assay was previously described (LaTemple *et al.*, 1996), and was based on the ability of the enzyme to transfer galactose from the sugar donor UDP-Gal to terminal N-acetyllactosaminyl residues on N-linked carbohydrate chains of asialofetuin. Fetuin is a protein obtained from fetal calf serum that carries three N-linked carbohydrate chains, on which the terminal LacNAc residues are capped by SA (Green *et al.*, 1988). The removal of these SA units exposes nine LacNAc residues that function as sugar acceptors. Synthesized α-gal epitope were identified by the subsequent binding of the monoclonal anti-Gal antibody M86 (Galili *et al.*, 1998), as measured by ELISA. Desialylation of fetuin was performed by incubation of fetuin in 50 mM sulfuric acid at 80°C for 2 h and subsequent repeated dialysis. Asialofetuin (20 μg/ml) in carbonate buffer pH 9.5 was used to coat microtiter ELISA plates. After blocking with 1% BSA in carbonate buffer the enzyme preparation at different dilutions in 100 mM 2-[N-morpholino]ethan sulfonate (MES) buffer (pH 6.2) containing 25 mM $MnCl_2$ and 1 mM UDP-Gal were added to the plate. After incubation at 37°C for 1 h, the plates were washed and the M86 monoclonal anti-Gal antibody was added and incubated at room temperature for additional 1 h. Subsequently, the plates were washed and incubated with HRP-conjugated anti-mouse IgM as secondary antibodies for 1 h at room temperature. After additional washing the color reaction with O-phenylenediamine was measured at 492 nm.

Purification of rα1,3 GT

The secreted rα1,3GT in 1 L supernatants from 3-day cultures was precipitated with high salt (50% ammonium sulfate) and spun at 30,000 × *g* for 30 min. The pellet was resuspended in 20 ml PBS containing 10 mM imidazole, and the resulting solution was passed through a 1-ml nickel-Sepharose column (Invitrogen) that was equilibrated with PBS/10 mM imidazole. After extensive wash with PBS containing 25 mM imidazole, the bound enzyme was eluted with MES buffer containing 250 mM imidazole. The concentration of the enzyme was measured by the colorimetric reaction (BCA system, Pierce) and the purity of the enzyme was determined by SDS–PAGE and western blot assay.

Analysis of purified rα1,3GT by SDS–PAGE and western blots

Affinity-purified α1,3GT (5 μg) was electrophoresed in 12% SDS–PAGE and then stained with Coomassie blue. For western blot analysis, 1 μg of purified rα1,3GT was electrophoresed on SDS–PAGE. The proteins were transferred to polyvinylidene difluoride membranes by semi-dry electro-blotting. The membrane was blocked overnight at 4°C in PBS with 3% defatted milk. The membrane was first incubated with anti-His antibody that binds to the $(His)_6$ tag, then with HRP-conjugated anti-mouse IgG. Color reaction was developed with diaminobenzidin (Sigma) substrate.

Analysis of synthesized α-gal epitopes on fetuin by SDS–PAGE and western blots

Fetuin (1 mg/ml) in MES buffer, pH 6.0, containing 25 mM $MnCl_2$ and 1 mM UDP-Gal, was incubated in three 0.1-ml aliquots with either neuraminidase (Sigma), or rα1,3GT (50 μg/ml) and neuraminidase (1 mU/ml). Subsequently, 8 μg of fetuin from each aliquot were subjected to SDS–PAGE and Coomassie blue staining. Two micrograms of the same samples were subjected to western blot analysis by staining with human anti-Gal isolated from normal serum of blood type AB donors (Galili *et al.*, 1984, 1985, 1987), followed by HRP–anti human IgG (Dako) or with the monoclonal anti-Gal M86 antibody (Galili *et al.*, 1998), followed by HRP-conjugated anti-mouse IgM as a secondary antibody.

Determining specific activity of rα1,3GT with cell surface glycoconjugates as acceptors

Because the produced rα1,3GT is to be used for the synthesis of α-gal epitopes on tumor cell membranes, the activity of the enzyme had to be determined with membrane-bound carbohydrate chains as acceptor, rather than with the disaccharide Galβ1-4GlcNAC (LacNAc) in solution, which is usually used for assaying native and recombinant α1,3GT activity (Basu and Basu, 1973; Blake and Goldstein, 1981; Betteridge and Watkins, 1983; Larsen *et al.*, 1989; Joziasse *et al.*, 1990; Henion *et al.*, 1994). This required the use of a novel assay designated ELISA inhibition assay, in which the total number of synthesized α-gal epitopes on membranes can be determined by the subsequent binding of the monoclonal anti-Gal antibody M86 (Galili *et al.*, 1998; Stone *et al.*, 1998; Tanemura *et al.*, 2000). This assay, which is analogous to radioimmunoassays, is a modification of the original assay for measuring α-gal epitope expression on glycoproteins by the use of the natural anti-Gal antibody (Thall and Galili, 1990).

As a source of membranes expressing carbohydrate chains that function as sugar acceptors for rα1,3GT, we used glutaraldehyde-fixed human red blood cells that were pretreated with neuraminidase (5 mU/ml). The red cells at a 10% concentration (i.e., 1×10^9 human red cells per ml) were mixed with rα1,3GT (50 µg/ml) in enzyme buffer (i.e.,100 mM MES buffer, pH 6.2, containing 25 mM $MnCl_2$ and 1 mM UDP-Gal) in a total volume of 200 µl. The mixture was incubated at 37°C for 2 h. After three washes with saline, the red cells were subjected to serial twofold dilutions in 100-µl aliquots of PBS containing 1% BSA. The red cells in each dilution were mixed with equal volume of monoclonal anti-Gal M86 at the final dilution of 1:100 of the antibody, that is, a concentration of the antibody that yields ELISA absorption results at the slope of the binding curve. The mixture was incubated overnight at 4°C with constant rotation to enable maximum binding of the antibody to α-gal epitopes. Subsequently, the cells and bound antibodies were removed by centrifugation, and the activity of free M86 antibody remaining in the supernatant was determined by ELISA with α-gal BSA as solid phase antigen, using HRP-conjugated goat anti-mouse IgM antibody as secondary antibody.

Untreated human red cells do not bind the antibody because they completely lack α-gal epitopes (Galili *et al.*, 1987). However, treated red cells that express *de novo* α-gal epitopes bind the antibody proportionally to the number of α-gal epitopes expressed on the cells. Therefore, the amount of free M86 antibody remaining in the supernatant is inversely proportional to the number of epitopes per cell. By comparing the binding of M86 to cells with known number of α-gal epitopes per cell (standard cells) to that of the antibody binding to the treated human red cells, it is possible to determine the number of α-gal epitopes synthesized *de novo* on the human red cells (Galili *et al.*, 1998). Rabbit red cells were used as the standard cells because they were previously shown to express $\sim 2 \times 10^6$ α-gal epitopes per cell (Galili *et al.*, 1998). Thus, if the treated human red cells are 10-fold less effective than rabbit red cells in the ELISA inhibition assay (i.e., 10-fold higher concentration of human red cells than that of rabbit red cells is required for 50% inhibition of anti-Gal M86 binding), the number of α-gal epitopes on the tested red cells is approximately 2×10^5 per red cell. Quantification of α-gal epitope expression on leukemia cells treated with rα1,3GT was determined by the same ELISA inhibition assay.

Synthesis of α-gal epitopes on human leukemia cells and ovarian carcinoma membranes

A total of 4×10^9 cells AML cells were received from a patient undergoing leukophoresis treatment. The cells were brought to a concentration of 1×10^8 cells/ml in the enzyme buffer that includes saline, 25 mM MES (pH 6.2), 25 mM $MnCl_2$, and 1 mM UDP-Gal. Ten milliliters of this cell suspension were incubated with rα1,3GT, (50 µg/ml), and neuraminidase (1 mU/ml) for 2 h at 37°C. Cells treated only with neuraminidase or only with α1,3GT served as controls. At the end of incubation the cells were washed twice with PBS containing 1 mM EDTA and twice with PBS. For demonstration α-gal epitope synthesis, the cells at 2×10^6 cells/ml were plated into ELISA wells as 50 µl per well. After the cells were dried in these microtiter wells, they strongly adhered to the wells. The plates were blocked with PBS containing 1% BSA. The α-gal epitopes on the cells were detected by the subsequent binding of HRP-conjugated BS lectin at various concentrations of the lectin. After additional washing the color reaction with O-phenylenediamine was measured at 492 nm, according to a previously described assay (LaTemple *et al.*, 1996).

Similar α-gal synthesis was performed with ovarian carcinoma tumor membrane homogenate at 200 mg/ml in the enzyme buffer. After completion of the α-gal epitope synthesis the membranes were washed, plated as 2 mg/ml in ELISA wells, and dried. The epitopes were subsequently detected by BS-lectin as described above for leukemia cells.

Flow cytometry analysis of α-gal epitope expression on AML cells

AML cells were incubated at a concentration of 1×10^6 cells/ml for 2 h at 4°C with 10 µg/ml FITC–BS lectin in PBS containing 1% BSA. Cells were then washed three times with PBS, fixed, and analyzed in a FACS Calibur flow cytometer (Becton Dickinson).

Western blot analysis of α-gal epitopes expression on leukemia cells

Twenty micrograms of enzymatically treated or control leukemia cell membranes were subjected to SDS–PAGE, blotted, and immunostained with human anti-Gal followed by HRP–anti human IgG as described above for immunostaining of fetuin on western blots.

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Abbreviations

α-gal BSA, BSA with linked synthetic α-gal epitopes; α-gal epitopes, Galα1-3Galβ1-4GlcNAc-R; α1,3GT, α1,3galactosyltrandferase; AML, acute myeloid leukemia; APC, antigen

presenting cell; BSA, bovine serum albumin; BS lectin, *Bandeiraea (Griffonia) simplicifolia* IB4 lectin; EDTA, ethylenediaminetetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LacNAc, N-acetyllactosamine; MES, 2-[N-morpholino]ethan sulfonate; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SA, sialic acid; rα1,3GT, recombinant α1,3GT; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAA, tumor-associated antigens.

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