Interaction of wild-type and variant mouse 3T3 cells with lectins from *Bandeiraea simplicifolia* seeds

(lectin resistance/cell membrane alterations/cytotoxicity)

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ABSTRACT An isolectin (BS I-B₄) derived from *Bandeiraea simplicifolia* seeds and specific for terminal α -D-galactopyranosyl groups was found to be cytotoxic to Swiss 3T3 mouse cells. After mutagenesis and selection with BS I-B₄, a variant clonal cell line resistant to both this isolectin and the α -D- and β -D-galactose-binding lectin abrin was isolated. The parental cell line showed homogeneous and noninteracting binding sites for BS I-B₄, whereas the variant cells exhibited a curved plot with a reduced number of binding regions. Another lectin, BS II, which is derived from the same seeds but specific for terminal N-acetyl-D-glucosaminyl groups, was cytotoxic to the variant but not the parental cells. These results suggest a possible lesion in the biosynthesis of cell surface structures resulting in the exposure of subterminal N-acetyl-D-glucosaminyl moieties in the variant line.

Carbohydrate-containing structures found on animal cell surface membranes play a vital role in the regulation of many important cellular events such as cell division, differentiation, immune response, intercellular communication, and normal and abnormal cell growth (1, 2). Lectins, which interact with specific carbohydrate moieties, have been used to probe the structural and functional roles of cell surface carbohydrates (3). In addition, several investigators have isolated cell mutants resistant to the cytotoxic action of some lectins (4-8). The 3T3 mouse cell line which exhibits contact inhibition of cell division has served as a model in investigations of cell surface properties after transformation. Mutants of 3T3 cells displaying altered membrane phenotypes would provide an opportunity to determine what changes, if any, in normal membrane structure are important to or associated with changes observed in transformed or tumor cells.

Murphy and Goldstein (9) described an isolectin, BS I-B₄, derived from *Bandeiraea simplicifolia* seeds, that is specific for binding α -D-galactopyranosyl groups. As an initial step in understanding the molecular organization of the membrane components of 3T3 cells, we report here information concerning the interactions of this lectin with Swiss 3T3 cells and a variant 3T3 cell line with impaired ability to bind this lectin.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 mouse fibroblasts were obtained from Myron Levine (Department of Human Genetics, University of Michigan Medical School). Wild-type and BS I-B₄-resistant variant cells were grown in a modified Eagle's medium (10) supplemented with either 5% or 10% fetal calf serum (GIBCO), penicillin (200 units/ml), and streptomycin (200 μ g/ml) or gentamicin sulfate (50 μ g/ml, Schering). Unless otherwise indicated, experiments were conducted at 36-37°C in a 5% CO₂-enriched air atmosphere.

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Mutagenesis and Selection. After 7 hr of incubation at 37°C of 10⁶ 3T3 cells in each of three 16-ounce (473 ml) glass bottles (Brockway), ethyl methanesulfonate was added to one bottle at a final concentration of 250 μ g/ml and to another at 400 μ g/ml. After 16 hr, the medium was replaced with growth medium for 7 hr. Mutagen treatment and medium changes were repeated as above. After 5 days of growth, 10⁶ cells from each bottle were equally divided and inoculated into two 6-cm plastic tissue culture dishes (Lux). The medium was replaced the next day with medium containing BS I-B₄ at 500 μ g/ml. Selection continued initially at this dose of lectin but was later decreased to 250 μ g/ml for a total of 13 days, during which sensitive cells rounded up and detached from the dishes. A single well-defined colony survived in the cell population whose progenitors were exposed to the higher dose of mutagen. After further cell multiplication and recloning, the lectin-resistant BS I-B₄^R cells, in contrast to the parental cells, were shown to have lost the ability to bind fluorescein-conjugated BS I-B₄.

Cytotoxicity of BS I-B₄. Wild-type 3T3 cells were seeded at a density of 2×10^5 per 3.5-cm dish. The following day, the medium was replaced with a medium lacking thymidine and containing 5% dialyzed fetal calf serum, 0.25 μ Ci of L-[¹⁴C]-leucine (3.33 mCi/mmol), 1 μ Ci of [³H]thymidine (0.4 mCi/mmol, New England Nuclear), and BS I-B₄ at either 50 or 100 μ g/ml. The plates were incubated at different time intervals and duplicate samples were harvested as follows. Detached cells were collected from the culture medium by centrifugation ($225 \times g$) and combined with cells removed by rinsing the dishes with phosphate-buffered saline (P_i/NaCl). The attached cells were digested with 1 ml of 0.1 M NaOH and this lysate was added to the combined cell pellet from the culture medium and rinsing solution. An aliquot (0.1 ml) of the cell digest was used to determine protein content and the remaining lysate was treated with an equal volume of 10% trichloroacetic acid. The acid-precipitable material was collected by filtration on glass fiber filters (Gelman) and assayed for radioactivity.

Cytotoxicity of Abrin. The sensitivity of 3T3 and BS I-B₄^R cells to the toxic lectin abrin (Calbiochem) was determined by two methods. For cell survival studies, 10⁵ 3T3 or BS I-B₄^R cells were inoculated into each 3.5-cm dish with medium containing 5% fetal calf serum. The following day, the medium was removed and replaced with medium containing different concentrations of abrin. After 4 days of culture, viable cells remaining attached to the culture dishes were removed with trypsin and counted. For radioisotope incorporation studies, 10⁵ 3T3 or BS I-B₄^R cells per 3.5-cm dish were incubated for 24 hr. The medium was replaced with medium lacking leucine and thymidine and containing 5% dialyzed fetal calf serum and

Abbreviations: BS I-B₄, isolectin from *Bandeiraea simplicifolia* seeds that is specific for α -D-galactopyranosyl groups; P_i/NaCl, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GlcNAc, N-acetyl-D-glucosamine.

different concentrations of aβrin. Duplicate plates were incubated for 3 hr before 0.25 μ Ci of L-[14 C]leucine (3.33 mCi/mmol) and 1 μ Ci of [3 H]thymidine (0.4 mCi/mmol) were added. Incubation was continued for an additional 3 hr. Reactions were stopped by removing the medium and rinsing twice with P_i /NaCl. Cells were removed from the dishes with 1 M NaOH after the monolayers were dried overnight. An equal volume of 10% trichloroacetic acid was added to the samples and the precipitates were removed by filtration on glass fiber filters.

Radiolabeling of BS I-B₄. BS I-B₄ was purified according to the procedure of Murphy and Goldstein (9) and brought to a concentration of 5.5 mg/ml in P_i /NaCl. Two milliliters of the lectin was added to 2 ml of 0.2 M borate buffer, pH 8.5. A 0.5-ml aliquot of *N*-succinimidyl[2,3- 3 H]propionate (specific activity, 66 Ci/mmol, Amersham, dissolved in benzene at 2 mCi/ml) was evaporated to dryness under a stream of nitrogen. The lectin solution was immediately added and the reaction vessel was placed on a rotary shaker at 2°C. After 60 min the reaction was terminated by the addition of 0.5 ml of 0.2 M glycine and the reaction vessel was agitated for an additional 20 min. The radiolabeled lectin was dialyzed exhaustively against P_i /NaCl, until less than 200 cpm/ml remained in the dialysate. The radiolabeled lectin retained greater than 90% of its hemagglutination activity when tested against human type B erythrocytes (University of Michigan Blood Bank) and had a specific activity of 16,500 cpm/ μ g of protein.

Autoradiography. Wild-type and variant cells grown on 22 $\times$ 22 mm glass coverslips were rinsed with serum-free medium and incubated for 30 min at 37°C with 0.5 ml of serum-free medium containing 3 H-labeled BS I-B₄ at 50 μ g/ml, with or without 10 mM methyl α -D-galactopyranoside. Coverslips were rinsed twice with serum-free medium and once with P_i /NaCl, fixed for 1–2 min in a 1:3 mixture of glacial acetic acid and absolute methanol, and mounted cell-side-up on microscope slides. Slides were coated with Kodak nuclear track emulsion NTB-3 and stored for 14 days at 4°C before they were developed and stained.

Cell Binding Studies. Cells were plated at densities between 1 and 2 $\times$ 10⁵ per 3.5-cm dish. Approximately 24 hr after plating, the medium was removed and the cells were washed twice with cold P_i /NaCl. Increasing amounts of radiolabeled BS I-B₄ (2–200 μ g/ml in P_i /NaCl) were added to separate dishes in duplicate and the cells were incubated for 2 hr at 2°C. The cells were subsequently washed three times with P_i /NaCl, and 1 ml of water was added to each dish. The cells were scraped from the dishes and the amount of cell-bound radioactivity was determined. Cell density determinations were performed in duplicate at each lectin concentration.

For each binding curve, duplicate dishes were assayed for nonspecific binding by using BS I-B₄ at 50 μ g/ml in 10 mM methyl α -D-galactopyranoside. The radioactivity present after washing these dishes was equal to that present after lectin incubation and washing of a dish containing no cells. These background cpm were subtracted from each point on the binding curve. Addition of 10 mM methyl α -D-glucopyranoside did not abolish the binding of radiolabeled lectin to the cells.

Cell Detachment Studies. Cells were inoculated at 5 $\times$ 10⁴ per 3.5-cm dish. After sufficient time for cell attachment (approximately 6 hr), the medium was removed and replaced with that containing different concentrations of lectin and sugars as indicated in the tables. At different time intervals, duplicate dishes of treated cells were rinsed with P_i /NaCl, and the cells that remained attached were removed with trypsin and combined with trypan blue for hemocytometer counting.

RESULTS

Occurrence of α -D-Galactopyranosyl Groups on the Surface of Swiss 3T3 Cells. Fluorescein isothiocyanate (FITC)-conjugated BS I-B₄ isolectin, at concentrations as low as 30 μ g/ml, bound to wild-type Swiss 3T3 cells to give patchy fluorescence of the cell membrane and processes. That FITC-BS I-B₄ binds specifically to α -D-galactopyranosyl groups on the surface of 3T3 cells was demonstrated by two different methods. (i) Methyl α -D-galactopyranoside (10 mM), a hapten-inhibitor of the BS I-B₄ isolectin, prevented the binding of FITC-BS I-B₄ whereas methyl α -D-glucopyranoside (20 mM), not an inhibitor of the BS I-B₄ isolectin, had no effect on FITC-BS I-B₄ binding. (ii) Pretreatment of 3T3 cells with coffee bean α -galactosidase (Boehringer/Mannheim, 1 unit/ml in McIlvaine phosphate/citrate buffer, pH 5.1) markedly decreased the ability of FITC-BS I-B₄ to bind to 3T3 cells, whereas identical pretreatment with *Aspergillus niger* β -galactosidase (Calbiochem, 2 units/ml in the same buffer) had no effect on FITC-BS I-B₄ binding.

Cellular Effects of BS I-B₄. Mouse 3T3 cells exposed to this lectin round up and ultimately detach from the surface of culture vessels. To quantitate this response, growing cells were incubated with graded concentrations of the lectin, and the viable cells (determined by trypan blue exclusion) that remained attached after rinsing with P_i /NaCl were counted (Table 1). BS I-B₄ was effective in altering the adhesion of 3T3 cells to the substratum. This action was sugar-specific because methyl α -D-galactopyranoside effectively inhibited cell detachment whereas methyl α -D-glucopyranoside did not. Determinations of mitotic index in the presence of lectin (data not shown) indicated that lectin-stimulated rounding-up of cells is not a consequence of mitotic induction. The basis for the lectin-induced cell detachment is not clear.

The incorporation of [3 H]thymidine and [14 C]leucine into DNA and protein was measured at various time intervals in 3T3 cells incubated with the lectin. After a 4-hr exposure to BS I-B₄ at 50 or 100 μ g/ml, no significant inhibition of protein or DNA synthesis was observed. After 8 hr, only slight differences from controls were evident, and after 24 hr, [14 C]leucine and [3 H]-thymidine incorporation was reduced to 52 and 58%, respectively, of control values (Fig. 1). These results can be compared with the protein and DNA synthesis inhibition profiles (Fig. 2)

Table 1. Viability of mouse 3T3 cells in the presence of graded levels of isolectin BS I-B₄

Treatment	% of control cell number				
	0 hr	12 hr	36 hr	60 hr	84 hr
Control	100	100	100	100	100
BS I-B ₄ (4 μ g/ml)	100	116	72.0	45.1	34.6
BS I-B ₄ (20 μ g/ml)	100	118	82.5	27.2	15.6
BS I-B ₄ (100 μ g/ml)	100	68.0	24.5	9.8	2.1
BS I-B ₄ (500 μ g/ml)	100	43.9	4.7	0.5	0.2
Buffer "500"	100	116	153	100	64.9
Buffer "100"	100	133	141	110	108
BS I-B ₄ (100 μ g/ml) + 10 mM Me α -D-Gal	100	121	128	114	93.6
BS I-B ₄ (100 μ g/ml) + 10 mM Me α -D-Glc	100	78.6	21.0	10.9	4.0

Cell number $\times$ 10⁻⁴ at 0, 12, 36, 60, and 84 hr was, respectively, 5.0, 4.4, 5.72, 12.24, and 28.06. Me α -D-Gal, methyl α -D-galactopyranoside; Me α -D-Glc, methyl α -D-glucopyranoside.

* Because the stock lectin concentration was 3.8 mg/ml, in treatments with high lectin concentrations the media were relatively more diluted with the dialysis buffer. In these two controls, the media were diluted to the same extent as in media containing lectin at 500 and 100 μ g/ml.

FIG. 1. Cytotoxicity of BS I-B₄ isolectin. Wild-type 3T3 cells were incubated with $[^{14}\text{C}]$ leucine, $[^3\text{H}]$ thymidine, and BS I-4 for different time periods. (A) $[^{14}\text{C}]$ Leucine incorporation. O, Control; Δ , lectin at 50 $\mu\text{g/ml}$; $\bullet$, lectin at 100 $\mu\text{g/ml}$. (B) $[^3\text{H}]$ Thymidine incorporation. O, Control; Δ , lectin at 50 $\mu\text{g/ml}$; $\bullet$, lectin at 100 $\mu\text{g/ml}$.

obtained after only 6-hr treatment with abrin, a toxic lectin that inhibits protein synthesis through ribosome inactivation (11). Treatment of 3T3 cells with abrin at 10^{-3} $\mu\text{g/ml}$ inhibited protein and DNA synthesis by greater than 50%. The different responses of cells to BS I-B₄ and abrin may indicate, but does not firmly establish, different modes of action for these two lectins.

Properties of BS I-B₄-Resistant Variant Cell Line. After mutagenesis with ethyl methanesulfonate and selection with BS I-B₄, a clone resistant to the action of this lectin was isolated. After growth for more than 4 months in the absence of selection, this clonal line remained completely resistant to the lectin at doses as high as 500 $\mu\text{g/ml}$ (data not shown). BS I-B₄^R cells displayed a slower growth rate than did the parental cells (Fig. 3), but this small difference could be explained by clonal variation and may not be related to the altered phenotype. As do normal 3T3 cells, BS I-B₄^R cells exhibited contact inhibition of growth.

Phase-contrast micrographs showed that BS I-B₄^R cells have an increased number of vacuoles, which could result from enhanced endocytotic activity. Others have presented evidence to indicate that some lectins can be internalized, presumably by pinocytosis (12–14). The rate of endocytotic activity was measured by following the uptake of $[^3\text{H}]$ inulin in normal and variant cells. The BS I-B₄^R cells displayed a slightly decreased

FIG. 3. Growth of 3T3 (O) and BS I-B₄^R (●) cell lines. On day 0, 5×10^4 cells were inoculated to each 3.5-cm dish in 1 ml of medium containing 10% fetal calf serum. Daily, cells from duplicate dishes were removed with trypsin and counted. Medium was changed every other day.

rate of inulin uptake compared to parental cells (data not shown). In addition, the fact that the BS I-B₄^R cells were also cross-resistant to abrin (Fig. 2) argues against the possibility of increased pinocytosis in these cells. It has been shown that both abrin and ricin can be taken up by mammalian cells, presumably by pinocytosis, and that the internalized toxin is involved in the inhibition of protein synthesis (7, 12).

If BS I-B₄ were being rapidly taken into the cell, then it should be detected near the cell membrane and within the cytoplasm after a short incubation. Autoradiographs prepared from cells grown on coverslips and incubated for 30 min at 37°C with ^3H -labeled BS I-B₄ showed (Fig. 4) BS I-B₄^R cells to contain fewer grains associated with the cells compared to parental 3T3 cells. The inclusion of 10 mM methyl α -D-galactopyranoside, a sugar-hapten inhibitor of the BS I-B₄ lectin, in the medium decreased the number of grains associated with parental cells to that observed with the variant line. Our data suggest that resistance of the variant cell line to BS I-B₄ is not due to an increased rate of endocytosis and subsequent intracellular digestion. The autoradiographic study suggests that, compared to parental cells, much less lectin is interacting with carbohydrates on the surface of BS I-B₄^R cells.

Binding studies were performed with radiolabeled BS I-B₄ to quantify the differences between the parental and variant cells. Initial studies to determine the time dependence of binding were performed at the lowest lectin concentration used for subsequent binding studies. After 2 hr at 2°C, only small

FIG. 2. Response of wild-type 3T3 (O) and BS I-B₄^R (●) cells to graded concentrations of abrin, expressed as % of control value. (A) Cell survival. (B) $[^{14}\text{C}]$ Leucine incorporation. (C) $[^3\text{H}]$ Thymidine incorporation.

FIG. 4. Autoradiographs of 3T3 (A and B) and BS I-B₄^R (C and D) cells incubated for 30 min at 37°C with ³H-labeled BS I-B₄ at 50 µg/ml, in the absence (A and C) and presence (B and D) of 10 mM methyl α-D-galactopyranoside. (×400.)

increases (<10%) were observed in the amount of lectin bound to the cells. The specifically bound lectin could subsequently be removed from the cell surface by the addition of 20 mM methyl α-D-galactopyranoside. This indicated that little or no internalization of the lectin occurred during the 2-hr incubation period. Therefore, 2 hr was chosen as the standard incubation time in all subsequent binding studies.

Fig. 5A shows the binding of BS I-B₄ to 3T3 cells, plotted according to Scatchard (15). The absence of curvature in this plot indicates that, over a 100-fold concentration range, the cell surface binding sites for BS I-B₄ appear to be homogeneous and noninteracting. From the slope of this line, a binding constant of $2.2 \times 10^7 \text{ M}^{-1}$ was calculated. If one assumes that, at saturation, one lectin molecule binds to each receptor site and that all receptor sites are accessible to the lectin, then calculations indicate the presence of 39×10^6 lectin-reactive sites per cell. The binding was specific; it could be inhibited by 10 mM methyl α-D-galactopyranoside but not by 10 mM methyl α-D-glucopyranoside. In contrast to the straight line observed with parental cells, a Scatchard plot of BS I-B₄ binding to the variant cells over the same concentration range yielded a biphasic plot (Fig. 5B). From the slopes of tangents constructed to this curve, binding constants of $2.3 \times 10^7 \text{ M}^{-1}$ and $2.6 \times 10^6 \text{ M}^{-1}$ were

FIG. 5. Binding of BS I-B₄ to parental and variant cells plotted according to Scatchard (15). [A], concentration of free lectin; r, mol of lectin bound per cell. (A) Lectin binding to parental cells at a density of 0.126×10^6 cells per 3.5-cm dish; (B) lectin binding to variant cells at a density of 0.174×10^6 cells per 3.5-cm dish. Lectin concentration varied from 2 to 200 µg/ml in 137 mM NaCl/5.4 mM KCl/5.0 mM phosphate buffer, pH 7.4/1.8 mM CaCl₂/0.8 mM MgSO₄.

Table 2. Viability of mouse 3T3 and BS I-B₄^R cells in the presence of different levels of BS II lectin

Treatment	% of control value at 84 hr	
	3T3	BS I-B ₄ ^R
Control	100	100
BS II (100 µg/ml)	99.7	0.52
BS II (500 µg/ml)	66.2	0.52
BS II (100 µg/ml) + 20 mM GlcNAc	90.0	87.0

calculated. The number of high-affinity binding sites at saturation was 4.3×10^6 per cell, approximately $1/10$ th that determined for parental cells at the same cell density. The sum of high- and low-affinity sites was calculated to be 9.8×10^6 per cell.

We have observed that BS I-B₄^R cells are cross-resistant to abrin (Fig. 2) and do not bind FITC-conjugated ricin (data not shown). Because these lectins bind to both α-D- and β-D-galactosyl groups (16), our findings imply that BS I-B₄^R cells have a decreased number of these groups on the cell surface. Other investigators (5, 17, 18) have found ricin-resistant cell lines with decreased N-acetyl-D-glucosaminyltransferase activity, suggesting that the lectin resistance may be due to incomplete biosynthesis of terminal D-galactose-containing glycoproteins. There is evidence (19–21) that in many glycoproteins N-acetyl-D-glucosamine (GlcNAc) is situated in a subterminal position to β-D-galactosyl units. Hence, if BS I-B₄^R cells were unable to add terminal D-galactose onto surface structures, these cells might contain increased amounts of terminal GlcNAc. To test this possibility, we used a second lectin (BS II) isolated from *B. simplicifolia* seeds that is specific for terminal N-acetyl-D-glucosaminyl units (22, 23). We found a strong binding of FITC-conjugated BS II to BS I-B₄^R cells and no observable fluorescence with parental 3T3 cells. We also examined the interaction of this agglutinin with growing 3T3 and BS I-B₄^R cells by determining the viability of attached cells at different time intervals. A summary of these data is shown in Table 2. It is clear that BS I-B₄^R cells, but not the 3T3 line, have D-GlcNAc residues available that can interact with BS II, leading to detachment from the culture vessel. Thus, the response of BS I-B₄^R cells to BS II is similar to that of 3T3 cells to BS I-B₄. This result suggests a possible lesion in the addition of D-galactosyl units onto D-GlcNAc residues of cell surface structures in the variant line.

DISCUSSION

The isolectin BS I-B₄ exhibits rigid binding specificity for the α-D-galactopyranosyl group (9), a linkage reported to occur with low frequency in mammalian glycoconjugates (24, 25). The 3T3 cells bind this lectin, demonstrating the occurrence of α-D-galactopyranosyl groups on their surface. We have taken advantage of this interaction to obtain a variant line resistant to BS I-B₄-mediated cytotoxicity.

The addition of BS I-B₄ to growing 3T3 cells noticeably affects their adhesion to the substratum and culminates in cell detachment. This dose-dependent response requires specific lectin binding to the cell surface because addition of methyl α-D-galactopyranoside, but not methyl α-D-glucopyranoside, to the culture effectively inhibits lectin-induced detachment. BS I-B₄ also inhibits the incorporation of [¹⁴C]leucine and [³H]thymidine into proteins and DNA, respectively, of 3T3 cells, although significant inhibition is not observed until after 24 hr of treatment. The mechanism by which macromolecular synthesis is inhibited is not yet clear.

When examined over the same lectin concentration range,

the parental and variant cells exhibited strikingly different binding curves. The parental cell line showed homogeneous and noninteracting binding sites for BS I-B₄. The variant exhibited a curved plot with only 1/10th as many high-affinity binding sites as were present in the parental cells. Because the binding studies were performed only over a 100-fold concentration range, perhaps not all classes of lectin binding sites were observable for each cell type. However, these binding curves do allow comparisons between parental and variant cells. With the variant cells we are able to detect a second class of binding regions for BS I-B₄.

Although a number of alterations could be responsible for this response, the evidence for cross-resistance to abrin, an α -D- and β -D-galactose-binding lectin, suggests a possible lesion involving terminal D-galactose units. This is strengthened, although not proved, by the observation that the variant line has newly exposed terminal D-GlcNAc residues not present on parental 3T3 cells. Several lines of evidence indicate that D-GlcNAc is often found subterminal to β -D-galactose in glycoproteins (19–21).

We find no significant change in exponential growth rate or in density-dependent growth inhibition. In this connection, it is interesting to compare our findings with the work of Pouyssegur *et al.* (26, 27) who selected from BALB/c 3T3 cells a variant line with impaired cell adhesion properties. The changed phenotype results from a lesion in the acetylation of D-glucosamine 6-phosphate leading to incomplete glycoprotein biosynthesis. This particular cell line displays additional properties associated with transformed cells but is nevertheless similar to parental cells in growth properties. Experiments with this variant line have helped identify properties associated with transformed cells that are not directly related to loss of growth control. By similar argument, further study of our variant 3T3 cell line lacking terminal galactosyl groups on its surface but exhibiting normal growth properties may put into new perspective studies by Roth and White (28) correlating changes in growth state with the galactosylation of cell surface acceptors. The characterization of cell mutants with defined alterations should provide information essential to the understanding of the role of surface components in the expression of aberrant phenotypes.

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