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Contribution of α-D-galactopyranosyl End Groups to Attachment of Highly and Low Metastatic Murine Fibrosarcoma Cells to Various Substrates

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There are much greater numbers of cell surface terminal, non-reducing α-D-galactorpyranosyl groups in highly malignant (metastatic) cells than are found in low malignant cells derived from the same murine fibrosarcoma. We have examined the contribution of these residues to attachment of the cells to various collagens and to plastic. Removal of these carbohydrate groups with α-galactosidase or blocking them with lectins from *Griffonia simplicifolia* seeds or with anti-blood group B antiserum all dramatically inhibited the attachment of both the highly malignant and the low malignant cells. Following removal with the enzyme, the α-D-galactopyranosyl end groups were rapidly resynthesized. This resynthesis was inhibited by tunicamycin, an inhibitor of de novo glycoprotein synthesis. This antibiotic also impaired cell attachment and, when used in addition to treatment with α-galactosidase, it inhibited cell attachment more than did treatment with the enzyme alone. The effects of all treatments on cell attachment were greater for the highly malignant than for the low malignant cells. With the latter cells, inhibition by lectin was seen only in the absence of serum, whereas the adhesion of highly malignant cells was affected in both the presence and the absence of serum. On their surface membrane the highly malignant cells express much more than do the low malignant cells of a glycoprotein that cross-reacts immunologically with laminin. The basement membrane glycoprotein laminin promotes cell attachment to collagen, and both glycoproteins contain terminal, non-reducing α-D-galactopyranosyl groups. Attachment of cells is a requirement for the formation of a metastasis, and thus the laminin-like molecule and the α-D-galactopyranosyl end groups (whether on the laminin-related moiety or on other cell surface molecules) may both be important for expression of the most malignant phenotype.

In previous studies we have isolated and characterized several lines of highly malignant and low malignant murine fibrosarcoma cells from an uncloned tumor [16, 24, 25, 28, 29]. The molecular basis for the differences between the highly and low malignant lines has not been delineated. It has recently been found, however, that the highly malignant cells react strongly with *Griffonia simplicifolia* I-B_4 isolectin (GS I-B_4), while reactivity of the low malignant cells is not discernible when live cells are examined by microscopy with fluorescein isothiocyanate (FITC)-conjugated isolectin [26]. The GS I-B_4 lectin is specific for terminal, non-reducing α-D-galactopyranosyl groups [15]. The highly malignant cells

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are killed by complement-mediated cytotoxicity in the presence of antibodies requiring α-D-galactopyranosyl end groups for binding (antibodies to human blood group B), while the low malignant cells are much more resistant [12, 13]. These findings suggest that there are great differences between these cells in the amount of cell surface α-D-galactopyranosyl end groups they express. While we do not know the biological importance of this difference, this is not the only model in which tumor cell populations with differing levels of terminal α-D-galactopyranosyl groups on their surface have been described. Stanley & Chu [20] isolated a cell line lacking α-D-galactopyranosyl end groups from the murine L-929 fibroblastic cells, which express this moiety. It was found that while the parent L-929 cells formed tumors upon inoculation into athymic mice, the variant line was non-tumorigenic. In view of these findings from two model systems suggesting a relationship between terminal α-D-galactopyranosyl groups and malignancy, we have attempted to probe the relationship between the expression of this carbohydrate on the cell surface and the biological behavior of the cell lines. In this report we describe the effects of three treatments directed against the α-D-galactopyranosyl moieties on the ability of the fibrosarcoma cells to attach to collagen and plastic substrates.

MATERIALS AND METHODS

Cells

We have previously isolated and characterized prototype highly malignant (1.0/L1) and low malignant (1.0/anti-B^r) murine fibrosarcoma cells [13, 14, 23, 24, 28, 29].

In the present study, the cell lines were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2/95% air and subcultured by trypsinization as necessary. For use in the various assays, cells were harvested by brief (less than 2 min) trypsinization (Trypsin 1 : 250, Difco, Detroit, Mich.). Compared with harvesting by scraping, trypsinization did not alter the relative adherence or staining patterns, although trypsinized cells adhered rather less rapidly. Trypsinization yielded close to 100% trypan blue-excluding cells, whereas scraping decreased viability. Prior to use in these studies, both cell types were found to be free of mycoplasma by growth in mycoplasma broth and growth on mycoplasma agar.

Determination of Terminal α-D-galactopyranosyl Residues

The presence of terminal α-D-galactopyranosyl groups on the surface of the highly malignant cells was demonstrated by using fluorescein isothiocyanate (FITC) conjugated GS I-B_4. This lectin has previously been shown to have strict specificity for terminal, non-reducing α-D-galactopyranosyl groups [15]. We have described the staining procedure previously [27]. As before, binding specificity was always checked by using the specific hapten, methyl-α-D-galactopyranoside. In preliminary experiments the non-binding sugar methyl-α-D-galactopyranoside (Sigma Chemical Co.) was used in the same way. Fluorescence levels were determined by two investigators independently, using standard fluorescence microscopic techniques and coded slides, and were scored between 0 (i.e., no fluorescence; cells barely or not perceptible) and 4+ (intense uniform fluorescence in 100% of the cells).

Adherence Assay

Cell attachment to plastic tissue culture dishes and to collagen-coated dishes was quantified as described before [4, 30], with some modifications: Cells (2×10^5 per dish) were plated in pre-

equilibrated RPMI 1640 medium supplemented as described in Results and placed in the culture incubator. Later, at various times, the non-attached cells were removed, and the attached cells were harvested with trypsin. The cells in each population were counted with a Coulter Counter, model ZB. During the longest incubation periods, attached cells can divide. Following incubation on uncoated plastic in the absence of serum, the attached cells are very resistant to trypsin. These two potential problems were avoided by counting only the non-attached cells in such groups. Type I collagen and type IV collagen were used to coat plastic dishes as previously described [26], except that 50 μg of collagen was deposited in each of the dishes, which were then sterilized under ultraviolet light for 2 h. Type I collagen prepared from mouse skin was kindly provided by Dr Sem H. Phan (Department of Pathology, University of Michigan). Type I collagen from rat tail (used only in the experiments in fig. 2) and type IV collagen from human placenta [3], were from Sigma Chemical Co.

Treatment with Enzyme and Inhibition of Protein Glycosylation

Green coffee bean α-galactosidase (EC 3.2.1.22) was used to remove the α-D-galactopyranosyl groups from the cell surface. The enzyme was obtained from Sigma Chemical Co. Prior to its use with viable cells, the enzyme was tested for activity against its specific substrate (α-D-galactopyranoside) and against several other glycoside substrates including β-D-galactopyranoside, α-D-glucopyranoside, *N*-acetyl-β-D-galactosamide, *N*-acetyl-β-D-glucosamide, α-L-fucoside and α-D-mannoside. The *p*-nitrophenyl derivative of each of these glycosides was used; they were purchased from Sigma Chemical Co. All assays were conducted at pH 6.0 in 0.05 mol/l phosphate buffer. A standard assay procedure described by Bosmann, Lockwood & Morgan [2] was used throughout, except that all of the reactions were carried out in a microtiter plate (final volume of 200 μl) and the results were read using a Titertek Multiscan (Flow Laboratories, McLean, Va.). The stock solution of enzyme contained 42 units of activity per ml of buffer (3.2 mol/l $(NH_4)_2 SO_4$). Against α-D-galactopyranoside, activity was detected within 30 min using 50 μl of a 1 : 100 000 dilution of the enzyme. In contrast, no significant activity was detected against any of the other substrates at a 1 : 100 final dilution of the enzyme stock (largest amount tested). The α-galactosidase preparation was also examined for protease activity. ^{125}I-hemoglobin was used as the substrate in a solid-phase protease assay, as described previously [22]. In 50 μl of the stock enzyme, protease activity equivalent to less than 2 ng of trypsin was detected. Use of green coffee bean α-galactosidase from Boehringer Mannheim gave similar results.

All treatment of cells with enzyme was done at 37°C. Treatment of cells with α-galactosidase was conducted at pH 6.0. Maximal enzyme activity was obtained at pH 6.0, and activity declined rapidly at a higher pH. Only about 25% of the maximum enzyme activity was observed at pH 6.25. Incubating the cells at pH 6.0 for 1–2 h produced no deleterious effects, as indicated either by increased staining with 0.08% trypan blue or by decreased plating efficiency and growth rates in subsequent proliferation assays in vitro. For these studies, harvested cells were washed twice in PBS (pH 6.0) and incubated in PBS (pH 6.0) containing 10% fetal bovine serum and 1.2 U/ml of α-galactosidase. Serum was included because omission resulted in decreased cell viability at pH 6.0, both with and without α-galactosidase present. Normally the concentration of cells was $1–2\times10^6$/ml when treated in this way. During incubation the cells were kept in plastic tubes and maintained in suspension by gentle rocking. Incubation was terminated by adding a large excess of growth medium buffered at pH 7.2. Cells to be assayed in RPMI 1640 medium with serum were washed once in such medium. Cells to be assayed in other media were washed four times in the appropriate medium. Control cells were treated in exactly the same manner, except that they were incubated without enzyme. Controls at both pH 6.0 and pH 7.2 were used with each experiment.

In some experiments, cells were treated with 0.5 μg/ml of tunicamycin (Sigma Chemical Co.), an inhibitor of protein glycosylation [21], alone or following treatment with α-galactosidase. When tunicamycin was used, it was included in the neutralization, washing and assay media. The trypan blue exclusion method showed that neither single nor combined treatment reduced cell viability as compared with when cells were kept at pH 6.0 (control) and treated with only α-galactosidase, respectively. We also obtained growth curves of cells that had been treated with tunicamycin (0.5 μg/ml of growth medium) for 24 h. By phase contrast microscopy the cells were adherent and well spread prior to addition of tunicamycin. No change in their morphology was seen after treatment for 5 h. After 24 h almost all the cells had become rounded, and some had detached from the plastic dish. When subsequently cultured in growth medium, the cells spread out again, and after a lag phase they had the same doubling time and reached the same saturation density as the control cells.

In certain experiments we used β-galactosidase (EC 3.2.1.23) obtained from Sigma Chemical Co. (Sigma grade VIII; purified from *E. coli*). The cells were harvested by brief trypsinization and incubated with β-galactosidase in PBS with 10% fetal bovine serum at pH 7.3, which is the optimum pH for this enzyme. Treatment was for 45 min, following which the cells were washed and analysed as following exposure to α-galactosidase. The *p*-nitrophenyl derivative of the specific substrate (β-D-galactopyranoside) was used in control experiments to check the activity of the enzyme preparation.

α-D-Galactopyranosyl-binding Lectins and Serum

The GS I lectin [6] was used to inhibit cell attachment. It was obtained as a gift from Dr Irwin J. Goldstein (Department of Biological Chemistry, University of Michigan). The GS I lectin is actually a mixture of five isolectins, each a tetramer composed of various combinations of the A and B subunits. One of these isolectins (GS I-B_4) has strict specificity for terminal, non-reducing α-D-galactopyranosyl groups [15]. To confirm that the effects observed with GS I were, in fact, due to the ability of the B_4 subunit to bind α-D-galactopyranosyl groups, we used the haptenic sugar, methyl-α-D-galactopyranoside, in conjunction with the lectin. Methyl-α-D-glucopyranoside served as a non-binding control sugar, and GS I-B_4 (from Sigma Chemical Co.) was used to inhibit cell attachment in some experiments. For reasons to be described later, hyperimmune human anti-A, anti-B, and anti-A,B sera were also used to interfere with cell attachment. These sera were purchased from four different companies: Ortho Diagnostics (Raritan, N.J.); Pfizer Diagnostics (Groton, Conn.); DADE, Division of American Hospital Supply Corporation (Miami, Fla.) and Biological Corporation of America (West Chester, Pa.).

RESULTS

Effects of Treatment with α-Galactosidase and Tunicamycin on Cell Attachment

We first examined the effects on cell attachment of treating the highly and low malignant cells with α-galactosidase at pH 6.0 for 45 min. After this treatment nearly 100% of the highly malignant cells showed no evidence of staining with GS I-B_4. In contrast, cells maintained at pH 6.0 but not exposed to enzyme showed the same staining pattern as cells maintained at pH 7.2 throughout. Nearly 100% of the cells were positive (+3 to +4), and this staining was eliminated when the cells were incubated with the lectin in the presence of the haptenic sugar, methyl-α-D-galactopyranoside. The non-binding sugar methyl-α-D-glucopyranoside did not affect staining. Greater than 95% of the cells incubated at pH 6.0 but not exposed to the enzyme were viable as indicated by the trypan blue stain exclusion method. This was identical with what was observed with cells maintained at pH 7.2 throughout. However, the treatment with enzyme did produce a slight increase in the number of dead cells. We routinely observed that 90–95% of the cells in the enzyme-treated groups were viable. It should be noted that 45 min was chosen as the time of incubation of the cells with the α-galactosidase after carefully weighing against each other enzyme effectiveness in removing cell surface α-D-galactopyranosyl groups and toxicity for the cells. In preliminary experiments, toxicity was evaluated with similar results with the trypan blue staining method, plating efficiency, and growth curves in vitro. Treatment for 30 min produced no toxicity but did not completely remove the α-D-galactopyranosyl residues from the highly malignant cells, although the degree

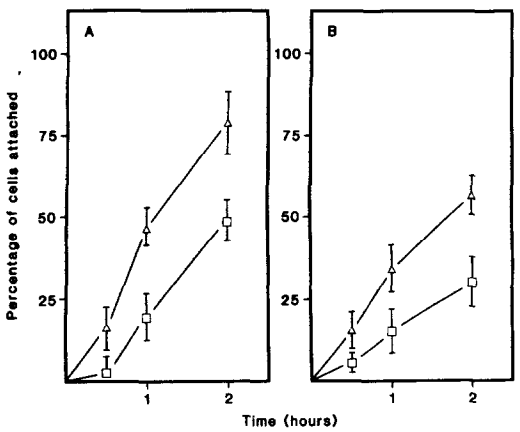

Fig. 1. Effect of treatment with α-galactosidase on attachment of highly malignant cells to collagen in presence of 10% fetal bovine serum. The cells were treated with the α-galactosidase and subsequently examined for ability to attach, as described in Materials and Methods. Cell attachment to type I collagen is shown in (*A*) and attachment to type IV collagen in (*B*). □, α-Galactosidase-treated cells; △, control cells incubated at pH 6.0, but not exposed to enzyme. The values shown are the means ± SD of two independent sets of experiments, each with duplicate samples.

of reactivity with FITC-GS I-B_4 was considerably diminished relative to control cells. On the other hand, treatment of the cells with the α-galactosidase for periods longer than 45 min produced an unacceptably high number of dead cells (greater than 10%). The low malignant cells had no detectable surface α-D-galactopyranosyl end groups as indicated by fluorescence microscopy with FITC–GS I-B_4. However, these cells must contain some of this sugar, because when extracts of the low malignant cells were separated by affinity chromatography on immobilized GS I, a small amount of material was retained that could be eluted with methyl-α-D-galactopyranoside [26]. The low malignant cells tolerated a 45-min treatment with α-galactosidase just as well as the highly malignant cells, judged by the same criteria.

Enzyme-treated highly malignant cells were examined for adherence to the collagen substrates in the presence of 10% fetal bovine serum. Fig. 1 shows that the enzyme-treated cells attached to both collagen types more slowly than the untreated control cells. Although not shown in fig. 1, the percentage of cells attached at 18 h was similar for both groups, indicating that the inhibition of attachment following treatment with enzyme was temporary. We observed (data not shown) that attachment to plastic dishes in the presence of 10% fetal bovine serum was also retarded after exposure to enzyme. Treatment of the cells with the α-galactosidase for 30 min inhibited attachment to plastic as well as to the collagen substrates. Not surprisingly, however, inhibition was not as great after 30 min of treatment as it was after 45 min. Untreated cells maintained at pH 7.2 throughout behaved as the control cells incubated at pH 6.0.

Since treatment of the highly malignant cells with α-galactosidase produced only a transient inhibition of cell attachment, we examined how soon after treatment the cells regained expression of terminal α-D-galactopyranosyl groups as assessed by their ability to stain with FITC–GS I-B_4 lectin. To do this, the cells were treated with enzyme in the normal manner and then replated in growth

medium and allowed to attach at 37°C. At various times, the attached cells were reharvested with trypsin and stained. No fluorescent cells were seen at very early time points. Detectable staining became evident in the attached cells as early as 1 h after the termination of treatment with enzyme. However, the staining was much weaker than with the cells not treated with enzyme. Essentially the same observations were made when, after incubation with enzyme, the cells were allowed to recover in suspension by rocking them gently in growth medium in plastic tubes at 37°C. The results of these experiments indicate that the α-D-galactopyranosyl residues on the cell surface and the ability to attach are both regained rapidly after treatment with α-galactosidase.

In addition to resynthesis of α-D-galactopyranosyl end groups on the cell surface, slowly acting serum-dependent mechanisms may have contributed to the regaining of ability to attach.

In further adherence experiments with α-galactosidase-treated cells, serum was omitted (fig. 2). Untreated cells and enzyme-treated cells were either not further modified or subjected to additional treatment with tunicamycin (0.5 μg/ml; see Materials and Methods), an inhibitor of protein glycosylation. Thus, control cells and cells treated in three different ways were compared for ability to attach to type I and type IV collagen substrates in the absence of serum. Fig. 2 shows that treatment of the highly malignant cells with α-galactosidase dramatically decreased both their rate and final (after 4 h) extent of attachment to both collagens. Inclusion of tunicamycin following treatment with enzyme partially prevented the recovery of ability to attach to both collagens. However, after first reaching a plateau, the attachment rate did increase with time, and this increase correlated with the recurrence of very faint fluorescence seen when incubating with FITC–GS I-B_4. The cells that had been treated only with enzyme regained α-D-galactopyranosyl groups more rapidly (data not shown). When the cells were treated with tunicamycin only, both the rate and the final extent of attachment to the collagens were decreased, but not as much as following the other two treatments (fig. 2).

Similar observations concerning attachment were made with the low malignant cells (fig. 2). Staining of these cells in parallel was not done. However, there were two especially noticeable differences between the attachment characteristics of the highly and low malignant cells in these experiments. First, the effects of treatment with α-galactosidase and tunicamycin separately or combined were greater with the highly malignant cells. Second, the inhibitory effect of exposure to tunicamycin appeared earlier with the low malignant cells.

Although no direct comparison was made of the attachment of the highly malignant cells in the presence and absence of serum following treatment with α-galactosidase, it was a reproducible finding that the inhibitory effect of the exposure to enzyme was clearly greater in the absence of serum. The effect of treatment with enzyme on the low malignant cells was tested only in the absence of serum.

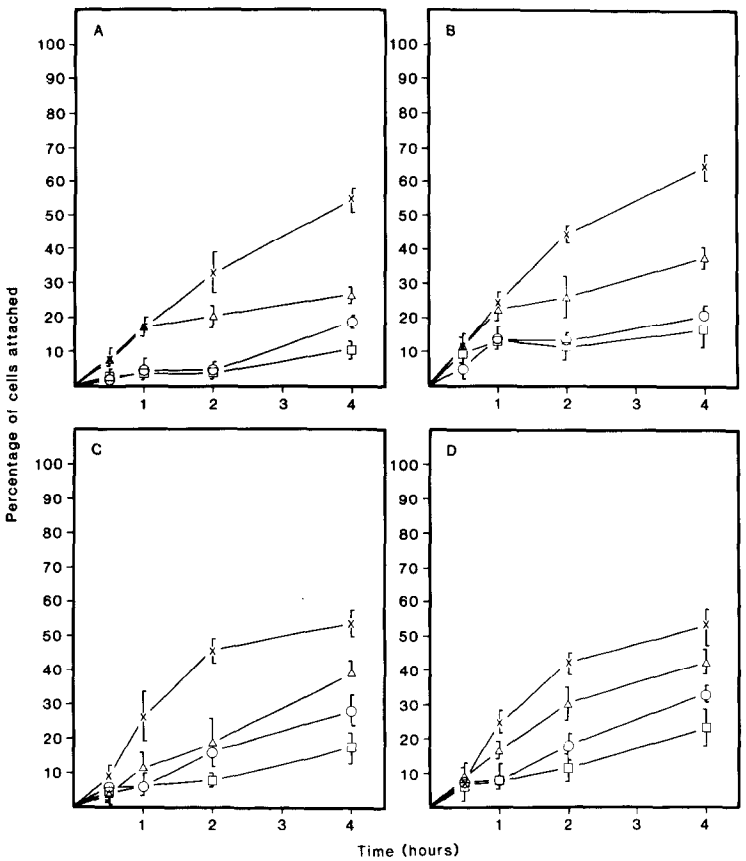

Fig. 2. Effect of treatment with α-galactosidase and/or tunicamycin on the attachment of (*A B*) highly; (*C, D*) low malignant cells to collagen in the absence of serum. Attachment to type I collagen is shown in (*A*) and (*C*), and to type IV collagen in (*B*) and (*D*). ×, Control cells; cells treated with ○, α-galactosidase; □, both α-galactosidase and tunicamycin; △, with tunicamycin only. The highly malignant and low malignant cells were assayed on separate occasions. Values shown are means ± SD of three independent sets of experiments, each with duplicate samples.

In other experiments, the cells were treated with β-galactosidase. Concentrations between 0.12 and 12 U/ml had no effect on cell viability, staining with FITC–GS I-B_4 or attachment to a variety of substrates (data not shown).

Inhibition of Cell Attachment by the α-D-Galactopyranosyl-binding Lectins GS I and GS I-B_4

We expected that the GS I lectin would inhibit the attachment of the highly malignant fibrosarcoma cells to the type I and type IV collagen substrates without any decline in activity with time. The results are shown in fig. 3. Whereas the control cells attached very rapidly to both collagen types in the presence of serum, the addition of GS I at 50 μg/ml completely and permanently inhibited attachment. In the absence of serum, cell attachment to both collagen types was

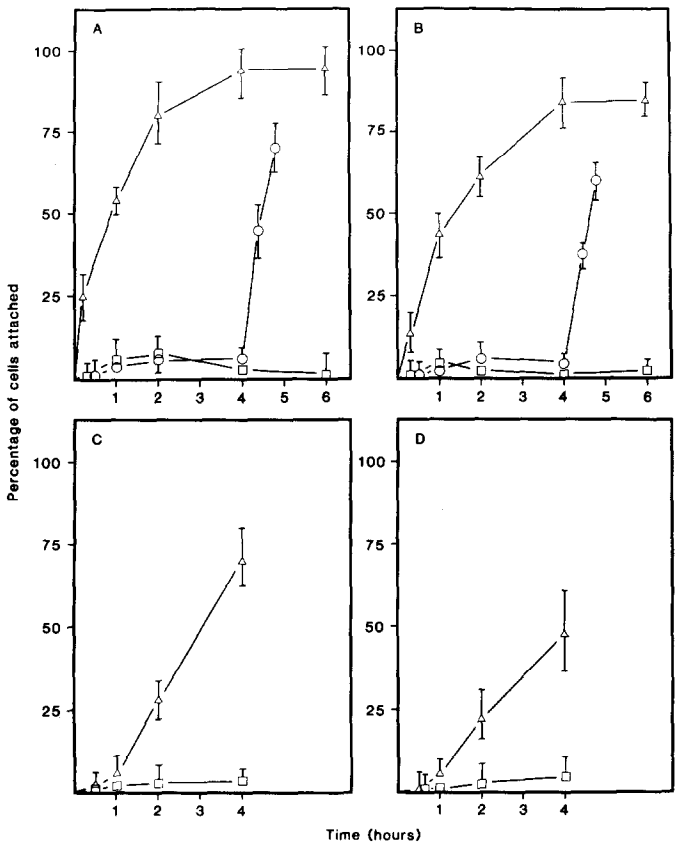

Fig. 3. Effect of GS I lectin on the attachment of highly malignant cells to collagen. Experiments depicted in (*A*) and (*B*) were carried out in the presence of 10% fetal bovine serum. Experiments shown in (*C*) and (*D*) were run under serum-free conditions. Cell attachment to type I collagen is shown in (*A*) and (*C*) and attachment to type IV collagen in (*B*) and (*D*). △, Control conditions; □, cultures in which 50 μg/ml of GS I lectin was used; ○, cultures in which 50 μg/ml of GS I lectin was added at time zero and in which 50 mmol/l of methyl-α-galactopyranoside was added at 4 h. The values shown in all panels (except for the methyl-α-D-galactopyranoside treatment) are means ± SD of three independent experiments, each with duplicate samples. Methyl-α-D-galactopyranoside was used in two experiments.

slower and less complete. However, the inhibitory effects of the lectin seen in the absence of serum were similar in magnitude to those observed in the presence of serum. Dose–response studies showed that cell attachment was blocked at concentrations of GS I as low as 10 μg/ml, but inhibitory activity fell away at lower concentrations (data not shown). The ability of methyl-α-D-galactopyranoside (50 mmol/l) to reverse the GS I-mediated inhibition of cell attachment (fig. 3) indicated that the effect of GS I was due to its α-D-galactopyranosyl-binding capacity. This also indicated that the lectin was not toxic to the cells. Further studies showed that the addition of 50 μg/ml GS I to cells 1 h after plating failed to release the already attached cells but did prevent cell spreading and caused cells that

Table 1. *Effect of GS I-B_4 lectin in combination with selected saccharides on the attachment of the highly malignant cells to type IV collagen-coated dishes in the absence of serum*[a]

	Percentage of cells attached
Control	86±1
GS I-B_4, 50 μg/ml + methyl-α-D-glucopyranoside, 50 mmol/l	11±1
GS I-B_4, 50 μg/ml + methyl-α-D-galactopyranoside, 50 mmol/l	90±1

[a] Cells were harvested and counted as described in Materials and Methods at a single time point 48 h after plating. All values represent the means ± SD of duplicate dishes in a single experiment. The assay was run three times.

were already spread to become round. The GS I lectin also completely inhibited the attachment of the highly malignant fibrosarcoma cells to plastic cell culture dishes in the presence of 10% fetal bovine serum. However, the lectin had no effect on the even much more rapid, presumably surface charge-mediated, attachment of the cells to the plastic culture dishes that occurred in the absence of serum (data not shown).

To firmly establish that the inhibition of cell attachment mediated by the isolectin mixture GS I was really due to its terminal α-D-galactopyranosyl-binding capacity and to show that inhibitory activity was retained even after prolonged incubation, other experiments were performed. The highly malignant cells were left on the type IV collagen substrate for 48 h in the absence of serum. The cells remained viable. Prior to addition of the cells, GS I-B_4 isolectin (50 μg/ml, which binds specifically to α-D-galactopyranosyl groups [15], had been added to some dishes along with methyl α-D-glucopyranoside (50 mmol/l) or methyl-α-D-galactopyranoside (50 mmol/l). Table 1 shows that in the presence of methyl-α-D-galactopyranoside, the GS I-B_4 lectin had no significant effect on cell attachment. However, when methyl-α-D-glucopyranoside was present, the lectin greatly inhibited cell attachment. Methyl-α-D-glucopyranoside by itself had no effect on cell attachment (data not shown). Thus, even during prolonged incubation the highly malignant cells did not overcome the attachment-inhibitory effect of specifically blocking α-D-galactopyranosyl end groups.

We also examined the effects of the GS I lectin on the attachment of the low malignant cells. Table 2 shows that in the presence of 10% fetal bovine serum, there was no significant effect on cell attachment to either of the two collagen substrates or plastic. By contrast, in the absence of serum, the GS I lectin significantly inhibited the attachment of these cells to both collagen substrates. Even here, however, the results were not as dramatic as those obtained with the highly malignant cells. Finally, we observed with the low malignant cells (as with

Table 2. *Effect of GS I lectin on the attachment of the low malignant cells to plastic dishes and to collagen-coated dishes*[a]

	Percentage of cells attached to		
	Plastic	Type I collagen	Type IV collagen
Serum-free	95±1	64±1	55±1
Serum-free + 50 μg/ml GS I	95±2	28±2	34±2
10% fetal bovine serum	95±1	82±3	77±1
10% fetal bovine serum + 50 μg/ml GS I	94±1	81±4	75±4

[a] Cells were counted at a single point 4 h after plating. All values represent the means ± SD of duplicate dishes in a single experiment. The assay was run twice.

the highly malignant cells) that treatment with lectin had no effect on the very rapid attachment to plastic in the absence of serum.

Inhibition of Cell Attachment by Antisera requiring α-D-galactopyranosyl End Groups for Binding

The human blood group B antigen contains terminal α-D-galactopyranosyl groups as part of its carbohydrate structure, whereas the group A antigen does not [32], and the human anti-blood group antibodies are directed toward the carbohydrate portion of the molecule [10]. We have previously shown that sera with anti-blood group B specificity (i.e., anti-B and anti-A,B) react with the cells containing α-D-galactopyranosyl end groups on their surface [13]. Whether these antibodies would affect cell adherence in vitro, however, was not known. In order to test this, we substituted the human hyperimmune typing sera (either anti-A, anti-B or anti-A,B) for fetal bovine serum in tissue culture medium and examined the ability of the highly malignant cells to attach to the type I and type IV collagen substrates. Several lots of each antiserum type (from four different manufacturers) were used. The results are shown in fig. 4. Although there was a tremendous variability from lot to lot with the anti-A sera, most of these sera allowed cell attachment to both collagen substrates. These antisera were examined on 2–3 occasions with little intra-lot variability from day to day. With the anti-B and anti-A,B sera present, clearly fewer of the highly malignant cells attached to either collagen type than in the presence of most of the anti-A sera. The results were most dramatic when type IV collagen was used. On this substrate, no appreciable cell attachment occurred with any of the 13 preparations of anti-B and anti-A,B sera present. This indicates specificity of cell attachment inhibition by those antisera binding to terminal α-D-galactopyranosyl-containing moieties. Fig. 4 also shows that cell attachment to the type IV collagen substrate was less than the attachment to the type I collagen.

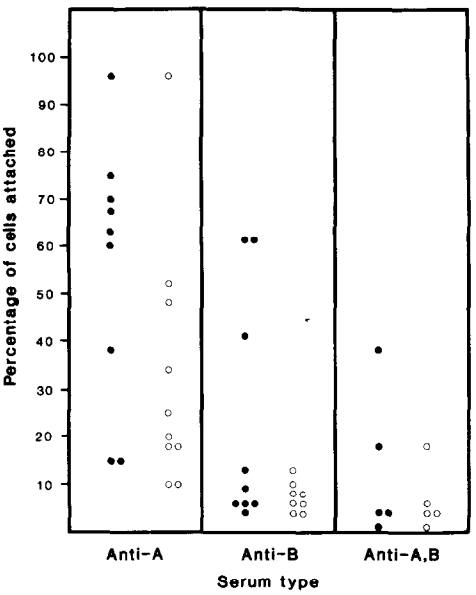

Fig. 4. Attachment of highly malignant cells to collagen in presence of hyperimmune human anti-A, anti-B, and anti-A,B sera. All values shown were obtained at a single time point, 4 h after plating the cells, in one single experiment. Each value represents a different lot of the hyperimmune serum. Lots of each serum type were examined on two to three occasions with little intra-lot variability. ●, Type I; ○, type IV collagen was the substrate.

The antisera directed against blood groups had similar effects on the attachment of the low malignant cells to the collagens. The anti-B and anti-A,B sera were very inhibitory compared with the anti-A sera, and with all of the sera cell adherence was clearly less with the type IV collagen substrate than with the type I collagen (data not shown).

DISCUSSION

In this study we have demonstrated that three treatments directed toward the α-D-galactopyranosyl end groups on the surface of murine fibrosarcoma cells inhibited the cells from attaching to collagen coated dishes and under some conditions also to plastic. These treatments included using (1) an enzyme which selectively cleaves the α-D-galactopyranosyl residues from the cell surface; (2) α-D-galactopyranosyl-binding lectins; and (3) human blood group-specific antibodies requiring α-D-galactopyranosyl groups for binding.

When cells were treated with the coffee bean α-D-galactosidase, the decrease in adhesiveness was accompanied by a loss in staining with the fluorescein-labelled lectin specific for α-D-galactopyranosyl groups. When the cells were incubated at 37°C in medium with 10% serum, they rapidly regained ability to stain with the lectin and concomitantly regained the ability to adhere to the substrate. However, when enzyme-treated cells were exposed to tunicamycin, both their ability to stain with the lectin and their ability to attach were further retarded. Tunicamycin inhibits *de novo* synthesis of the core oligosaccharide, but allows terminal regly-

cosylation of a core oligosaccharide already bound to a polypeptide chain [21]. Limited return of α-D-galactopyranosyl end groups and ability to attach were in fact observed even in the presence of tunicamycin. In contrast to these results, the enzyme specific for β-D-galactopyranosyl groups had no effect on cell staining or attachment. All in all, this is solid evidence that terminal α-D-galactopyranosyl groups are essential for cell attachment in this system.

The attachment experiments involving the lectins and the antibodies also suggested that the α-D-galactopyranosyl groups contribute to this cell function. With both agents, treatment of the highly malignant cells dramatically and specifically inhibited attachment to the collagen substrates. While it cannot be absolutely excluded that lectin and antibody, once they have bound specifically to the cells, are interfering with cell attachment simply by steric hindrance, the treatment with lectin had no effect on the ability of the cells to attach to plastic culture dishes in the absence of serum.

In contrast to these results with the highly malignant cells, the same treatments were, taken together, much less effective with the low malignant cells. These cells contain far fewer surface α-D-galactopyranosyl residues than the highly malignant cells [14]. This suggests that mechanisms which are, at least in part, independent of α-D-galactopyranosyl end groups play a greater role in the attachment of the low malignant cells than of the highly malignant cells. The existence of multiple attachment mechanisms in the former cells would not be surprising. Several studies have documented the existence of multiple attachment mechanisms in various cell types [5, 7, 9, 17, 31].

The molecular role of the terminal α-D-galactopyranosyl groups important for attachment of the fibrosarcoma cells to collagen remains to be determined. It is tempting to speculate that the existence of this sugar on the highly metastatic cells is due to the presence of the laminin-like moiety on these cells, since this is consistent with several documented facts. It is known that laminin contains terminal α-D-galactopyranosyl residues as part of its carbohydrate structure [18]. It is also known that the highly malignant cells used in this study express on their surface a substance which reacts with antibodies made to murine laminin [12, 26]. In light of these findings it is reasonable to suggest that attachment of the highly malignant cells is mediated, at least in part, by the laminin-like molecule and that the removal of the sugar residues on this glycoprotein or the addition of lectins or antibodies that bind to these carbohydrate residues disrupt the molecule's function. Perhaps the sugar is at the active attachment site. On the other hand, it may be that the sugar serves to maintain the proper conformation of the molecule. Additional work is necessary to determine if either of these ideas is correct. It is also possible that the cell surface terminal α-D-galactopyranosyl residues influence cellular adhesiveness independently of the laminin-like glycoprotein. This may be true particularly in the case of the low malignant cells, which do not express laminin-like molecules on their surface [12]. Interestingly, our recent studies [8] using high resolution two-dimensional electrophoresis demonstrated

that the protein patterns of the highly malignant and low malignant fibrosarcoma cells were very similar overall; only a very few differences existed.

Several studies have shown that other sugar moieties, particularly sialic acid, promote cell-to-cell and/or cell-to-substrate adhesion [1, 11, 19, 33]. While the mechanism by which sialic acid enhances adherence is not known, sialic acid levels (and relative adhesiveness) have been positively correlated with metastatic activity in murine tumor cells [34]. Thus, it seems possible that terminal α-D-galactopyranosyl groups and sialic acid groups may both contribute to the expression of the malignant phenotype by promoting ability to attach. The possibility of such a role for the α-D-galactopyranosyl end groups is not contradicted by the finding that the three treatments directed against these groups all inhibited attachment of both the highly and low malignant cells. Although ability to adhere is not a sufficient condition for metastatic capacity, it is probably a necessary condition. These sugar groups mediated cell attachment to type I and type IV collagen without any consistent preference for one over the other, and under some conditions also to plastic. Thus, the carbohydrate group studied here may be part of broadly active attachment-mediating structures found in various cell types.

In summary then, these studies show clearly that treatments removing or blocking the α-D-galactopyranosyl end groups dramatically decrease the attachment of the highly malignant murine fibrosarcoma cells to dishes coated with collagen of type IV (found in basement membranes) and type I (the collagen most widespread in interstitial tissue). This suggests that the terminal α-D-galactopyranosyl groups are involved in the attachment process, although their exact role is not known. The same treatments also decrease the attachment of the low malignant cells but only under particular conditions. Alternative attachment mechanisms may be more important in these cells than in the highly malignant cells. The differences in expression of cell surface terminal α-D-galactopyranosyl groups and in attachment that we have found between the cell types used here may be related to the dissimilarity in the capacity of metastasis identified previously in the same cells [25, 28, 29].

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