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Galectin-1 is expressed by thymic epithelial cells in myasthenia gravis

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Galectin-1, a member of a family of carbohydrate binding proteins, is synthesized by thymic epithelial cells in normal juvenile thymus, and mediates adhesion of immature T cells to thymic epithelium. Because cell adhesion molecules are proposed to play a role in the thymic hyperplasia and neoplasia seen in the autoimmune disease myasthenia gravis, we examined the expression of galectin-1 in myasthenic thymi. We detected abundant galectin-1 expression in thymic epithelial cells in 27 hyperplastic and neoplastic thymi from patients with myasthenia gravis. Primary cultures of neoplastic epithelial cells from a thymoma continued to express galectin-1, and bound immature T cells; T cell binding was inhibited by the addition of the β-galactosides lactose and thiodigalactoside, suggesting that galectin-1 on the thymoma cells and a saccharide ligand on the T cells participated in cell-cell adhesion. Expression of galectin-1 by thymic epithelial cells may play a role in the thymic pathology seen in myasthenia gravis.

Keywords: galectin-1, myasthenia gravis, thymus, thymoma, T cell

Introduction

Myasthenia gravis is a neuromuscular disorder characterized by fatigue and weakness of voluntary muscles, associated with circulating autoantibodies directed against the neuromuscular junction acetylcholine receptors [1, 2]. The thymus appears to have a role in the pathogenesis of myasthenia gravis, as thymic abnormalities are found in the majority of affected patients. The thymus normally involutes during adolescence; in myasthenia gravis, approximately 65% of patients have thymic hyperplasia, and an additional 10–15% develop thymoma [3]. Thymectomy often results in the improvement of myasthenic symptoms [4–6], even in patients without neoplastic disease [7]. A number of studies have documented disruption of the thymic microenvironment in non-neoplastic myasthenic thymi. Alterations in the cell surface phenotype of the thymocytes [8–10], and morphological and functional abnormalities of the thymic epithelial cells [11–13], have been described in myasthenic thymi.

The interaction of thymocytes with thymic epithelial cells has been shown to be an essential step in normal T cell development [14]. We have recently found that galectin-1, a member of a family of highly conserved carbohydrate binding proteins [15–17], is expressed by normal human thymic epithelial cells isolated from neonates and children [18]. Galectin-1 mediated the interaction of immature T cells to thymic epithelial cells by binding to specific oligosaccharide ligands on T cell surface glycoproteins. Galectin-1 has also been identified in other lymphoid tissues, including lymph node [19] and spleen [20]. In addition to participating in cell-cell and cell-substrate adhesion [21], galectin-1 has been proposed to have an immunoregulatory function. Administration of galectin-1 was therapeutic and prophylactic in a rabbit model of experimental autoimmune myasthenia gravis [22], and in a rat model of experimental autoimmune encephalitis [23]. The authors suggested that exogenous galectin-1 prevented T cell sensitization to autoantigens, perhaps by disrupting trafficking to lymphoid organs.

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Given the alterations in thymic epithelial cells which have been described in myasthenia gravis, it was of interest to examine the level of expression of galectin-1 by epithelial cells in myasthenic thymi from adult patients. We found that epithelial cells in both hyperplastic and neoplastic myasthenic thymi expressed high levels of galectin-1. In addition, primary cultures of neoplastic epithelial cells from a thymoma specimen continued to express galectin-1 in culture, and demonstrated carbohydrate-mediated adhesion of immature T cells in an *in vitro* binding assay. These results suggest a role for galectin-1 in recruitment or retention of thymocytes in the thymi of myasthenic patients, which may contribute to the pathogenesis and the morbidity of this disease.

Materials and methods

Patient samples

Twenty-four paraffin-embedded thymectomy specimens were retrieved from the Surgical Pathology archives at UCLA Center for Health Sciences. An additional three samples were obtained as fresh tissue during the course of this study.

Immunohistochemical analysis

Six μm paraffin sections of thymus tissue were blocked with 1% bovine serum albumin (BSA, Sigma) in 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4 (PBS) for 20 min at room temperature. Polyclonal rabbit antiserum to galectin-1 [15], or galectin-1-specific antiserum which had been adsorbed on galectin-1-Sepharose beads (as a negative control), were diluted 1:1000 in PBS containing 1% BSA, and incubated with the slides overnight at 4 °C. Bound primary antibody was detected by the addition of goat anti-rabbit antiserum conjugated to horseradish peroxidase (Bio-Rad) diluted 1:1000 in PBS. Bound secondary antibody was detected by the addition of the chromogenic substrate AEC (Peroxidase Chromogen kit, Biomeda) according to the manufacturer's directions, except that colour development was performed for 10 min at 37 °C.

Western blot analysis

Thymic stroma was obtained from freshly excised thymomas from two patients with myasthenia gravis. The stromal tissue was separated from the thymocytes by mechanical dissociation. Thymic stroma and cultured epithelial cells were homogenized in a tissue grinder (Wheaton) in two volumes of 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.3 (Tris-buffered saline, TBS) containing 1% Triton CF-54 and 0.2 M lactose. The tissue homogenate was added to an equal volume of TBS containing 1 mM phenylmethylsulfonyl fluoride, 5 mM $CaCl_2$ and 0.5% NP-40. The samples were sonicated in a water-bath sonicator, then rotated for 2 h at 4 °C. The samples were centrifuged at 14 000 × **g** for 2 min in an Eppendorf model 5415 microfuge to pellet insoluble material. The supernatants were concentrated using a Centricon 10 microconcentrator (Amicon), and protein content of the samples determined with the BCA protein assay (Pierce).

Samples were subjected to SDS-PAGE in a 10% acrylamide gel, and blotted onto 0.2 mm nitrocellulose membranes. The nitrocellulose was blocked in PBS containing 5% nonfat dry milk for 30 min at room temperature. The polyclonal rabbit antiserum to galectin-1, diluted 1:1000 in PBS with 1% nonfat dry milk, was added for 4 h at room temperature. After three washes with PBS with 0.05% Tween-20, the blot was incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (1:1000 in PBS) for 2 h at room temperature and washed as above. Bound antibody was detected with the chromogenic substrate 4-chloro-1-napthol (Sigma).

Primary culture of thymomas

Cultures of neoplastic thymic epithelial cells from fresh thymoma specimens were initiated and propagated as described for normal juvenile human thymic epithelial cells [24], except that no 3T3 feeder cells were used. Confluent cultures were obtained in approximately 3 weeks. At various stages of subculture, galectin-1 expresssion was examined by Western blot analysis.

Adhesion assay

One day prior to the assays, the cultured thymoma cells were passaged into 24 well culture dishes (Costar) (5×10^5 cells per well). MOLT-4 T lymphoblastoid cells (2×10^5 cells ml^{-1}) were labelled overnight at 37 °C with 5 μC ml^{-1} of [^{3}H]-thymidine (25 Ci $mmol^{-1}$, Amersham) [18]. After labelling, the MOLT-4 cells were washed twice in RPMI 1640 containing 10 mM HEPES and 3% BSA (binding medium), to yield a final cell concentration of 10^5 cells ml^{-1}.

The thymoma cell monolayers were washed twice in binding medium prior to the addition of 0.5 ml (5×10^4 cells) of labelled MOLT-4 cells per well. The final ratio of MOLT-4 cells to thymoma cells was 1:10. The plates were incubated at 37 °C for the indicated times. Nonadherent MOLT-4 cells were removed by gentle aspiration, and the wells were washed three times with binding medium. Adherent cells were collected by the addition of 0.2% trypsin, 0.02% EDTA in PBS. Nonadherent cells, washes and adherent cells were added to 5 ml of Ecoscint (National Diagnostics) and the samples counted in a Beckman model LS 6000E liquid scintillation counter. Control wells contained labelled MOLT-4 cells in the absence of thymoma cells. The total number of counts bound was calculated as (cpm $\text{adherent}_{\text{sample}}$–cpm $\text{adherent}_{\text{control}}$) = (cpm bound). The

percent of cells bound was calculated as (cpm bound)/(cpm bound + free). All assays were performed in duplicate. For saccharide inhibition studies, the thymoma cells were incubated with 0.5 ml of 0.1 M solutions of the indicated saccharides (D-stereoisomers) in binding medium for 30 min at 37 °C. This solution was removed prior to the addition of the labelled MOLT-4 cells in binding medium containing the indicated saccharide.

Results

Expression of galectin-1 in thymectomy specimens from patients with myasthenia gravis

We performed immunohistochemical analysis, using a polyclonal rabbit antiserum against human galectin-1, on thymic specimens from 27 patients who had undergone thymectomy for myasthenia gravis. Nineteen specimens were described as hyperplastic, while a diagnosis of thymoma was made on the other eight specimens (Table 1). The patients ranged in age from 13–61 years, and were predominantly female.

All of the specimens demonstrated strong reactivity with the antiserum to galectin-1 (Fig. 1A–D). In control specimens incubated with adsorbed serum, no immunoreactive material was detected (Fig. 1E). In the hyperplastic thymi, reactive cells were demonstrated in both the cortical and medullary regions of the thymus. Based on their appearance, size and distribution, the reactive cells in the hyperplastic thymi are thymic epithelial cells; we have previously demonstrated that galectin-1 positive cells in the thymus are epithelial cells, by double-label immunofluorescence studies with anti-cytokeratin and anti-galectin-1 reagents [18]. The epithelial cells expressing galectin-1 in the cortex and medulla were discrete from the numerous small thymocytes which did not react with the specific antiserum (Fig. 1A,B). Immunoreactive material was detected on the epithelial cell surface, as well as in the cytoplasm. In addition, long processes emanating from the epithelial cells and interdigitating between the small thymocytes were also visualized, suggesting that galectin-1 on these cellular processes could contact numerous thymocytes.

Thymomas are tumors of neoplastic epithelial cells, with varying numbers of non-neoplastic thymocytes admixed with the neoplastic epithelium. All eight of the thymomas examined demonstrated marked reactivity with the anti-galectin-1 antiserum, with abundant staining of the sheets of epithelial tumour cells (Fig. 1C,D). As seen in the hyperplastic thymi, the infiltrating lymphoid cells did not react with the anti-galectin-1 antiserum. These results demonstrate that neoplastic transformation of the epithelial cells did not result in loss of galectin-1 expression; indeed, reactivity of the thymoma cells with the galectin-1 antiserum was equal to or greater than that seen in hyperplastic and non-neoplastic juvenile thymi (Fig. 1A,B,F).

Table 1. Pathological description of the thymectomy specimens examined for galectin-1 expression

Diagnosis	*Number*	*Age range (mean)*	*Gender, F:M*
Hyperplastic	19	16–48 (28)	19:0
Thymoma	8	13–61 (39)	5:3

Previous studies have demonstrated that corticosteroid administration increases galectin-1 expression in cultured cells [25]. While seven of the patients examined in this study were receiving corticosteroid therapy at or immediately prior to the time of thymectomy, galectin-1 was detected by immunohistochemical staining in thymus tissue from all 27 patients; there was no qualitative difference in the level of galectin-1 expression between the samples from the treated patients and the samples from patients not receiving corticosteroid treatment. Thus, the presence of galectin-1 in the thymi of the myasthenic patients did not appear to result from steroid therapy.

To confirm that the immunoreactive material which we observed in these specimens was galectin-1, we obtained fresh tissue from two thymoma specimens at the time of thymectomy. The thymic stroma was separated from the thymocytes by mechanical dissociation, and subjected to Western blot analysis using the anti-galectin-1 antiserum. As shown in Fig. 2A, both of the stroma specimens contained an immunoreactive band of M_r 14 kDa, the size of the galectin-1 monomeric subunit. This material co-migrates with purified recombinant galectin-1, seen in lane 3. In addition, a band at M_r 28 kDa is seen in the lanes; this material represents dimeric galectin-1, and is also seen in the control lane containing purified recombinant galectin-1. The two galectin-1 subunits dimerize via non-covalent, hydrophobic interactions, rather than by disulfide cross-linking, and thus do not completely dissociate during SDS-PAGE, even on reducing gels [15].

Primary cultures of thymoma cells continue to express galectin-1

To determine if the neoplastic thymic epithelial cells would propagate and continue to express galectin-1, one patient specimen containing a thymoma was minced aseptically and placed in culture. Unlike non-neoplastic thymic epithelial cells [18, 24], the thymoma cells grew in culture without requiring irradiated 3T3 cells as feeder cells. The primary culture was subcultured seven times, and samples of the cells at several passages were assayed for the production of galectin-1 by Western blot analysis. As seen in Fig. 2B, an immunoreactive band at M_r 14 kDa which co-migrated with purified galectin-1 monomer was detected in all of the primary cultures examined, and was

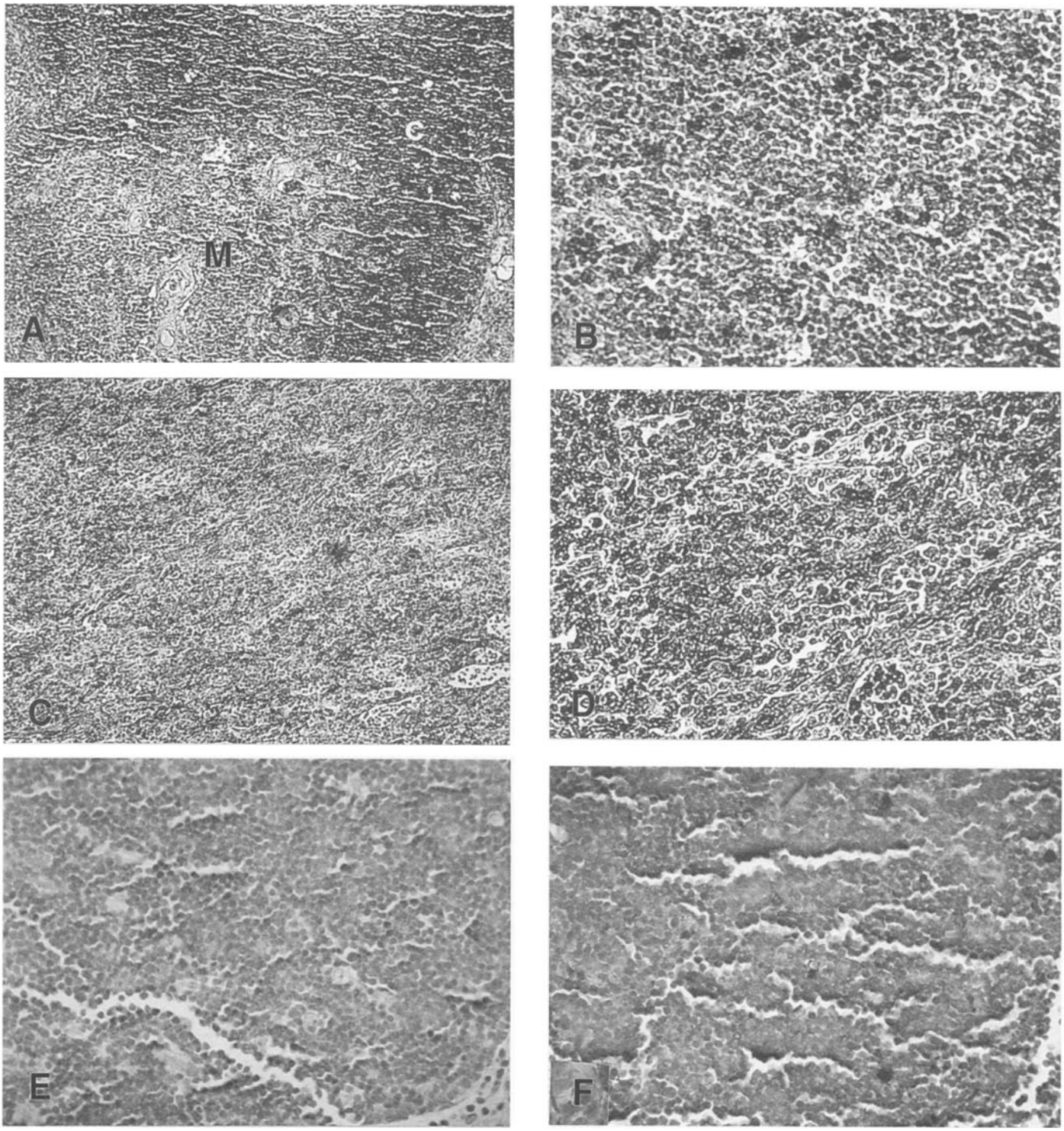

Figure 1. Expression of galectin-1 in hyperplastic and neoplastic thymectomy specimens from patients with myasthenia gravis. Formalin-fixed, paraffin embedded sections of thymus specimens were incubated with a polyclonal rabbit antiserum raised against recombinant human galectin-1. The sections were counterstained with hematoxylin. Representative sections of hyperplastic thymus (panels A and B) and thymoma (panels C and D) are shown. In panel A, reactive cells are present in both the cortical (C) and medullary (M) regions of the thymus. A cortical area is seen at higher power in panel B. Panel C demonstrates the sheets of neoplastic epithelial cells of a thymoma admixed with non-neoplastic thymocytes. The interconnecting network of malignant epithelial cells is evident in panel D. Control sections were stained with anti-galectin-1 antiserum which had been pre-adsorbed on galectin-1-Sepharose (panel E). A section of non-neoplastic thymus from a two-year old child, stained with anti-galectin-1 antiserum, is shown for comparison (panel F). Panels A, C × 100: panels B, D, E, F × 250.

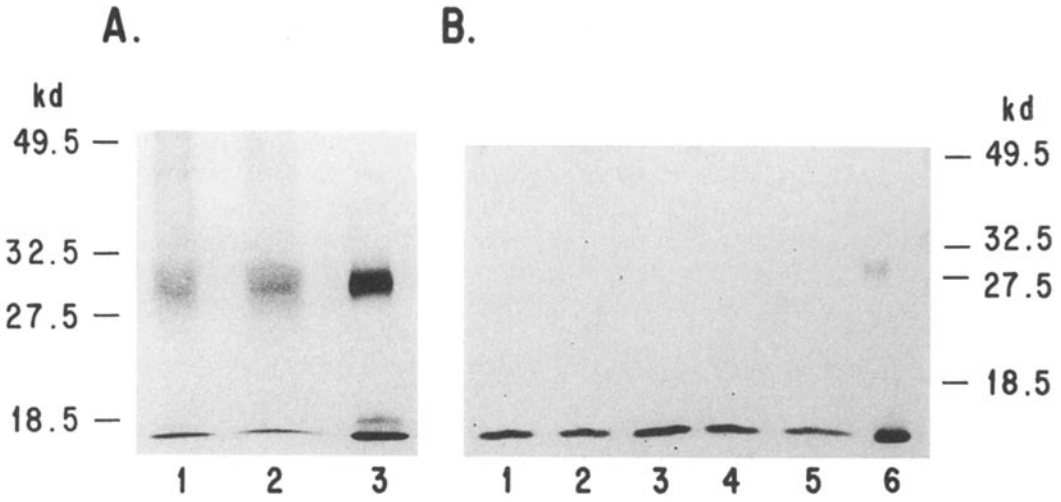

Figure 2. Expression of galectin-1 in thymoma specimens and in cultured thymoma cells, detected by Western blot analysis. A) Extracts of two thymoma specimens (lanes 1 and 2) and purified, recombinant galectin-1 as a positive control (lane 3). B) Four separate primary cultures of neoplastic thymoma cells after the first round of passage (lanes 1–4) and one sample after the fifth round of passage (lane 5), and purified, recombinant galectin-1 (lane 6).

still abundant in cells after five passages. These data demonstrated that the thymoma cells continued to express galectin-1 after several rounds of passage *in vitro*.

β-galactosidase inhibit the binding of immature T cells to cultured thymoma cells

The expression of galectin-1 by the cultured thymoma cells suggested that these cells would bind immature T cells via oligosaccharide ligands present on the lymphocyte cell surface. To examine this, monolayers of the cultured thymoma cells were assayed for the ability to bind MOLT-4 T lymphoblastoid cells, which have a cell surface phenotype similar to that of cortical thymocytes [18, 26, 27]. In addition to examining the ability of the MOLT-4 cells to bind to the thymoma cell monolayer, saccharides were added as inhibitors to the adhesion assay, to determine whether the binding which we observed was carbohydrate dependent.

As shown in Fig. 3A, specific binding of the MOLT-4 cells to the cultured thymoma cells was apparent after a

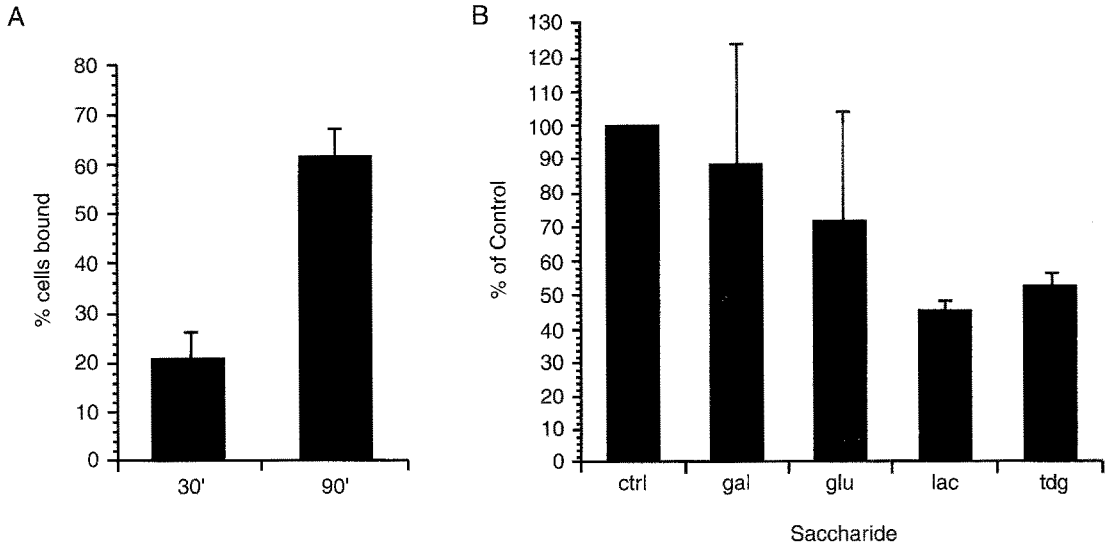

Figure 3. MOLT-4 cells bound to the cultured thymoma cells, and binding was inhibited by β-galactosides. A) Cultured thymoma cells were incubated with radiolabelled MOLT-4 cells for 30 and 90 min at 37 °C, and the percent of cells bound determined as described in Experimental Design. B) MOLT-4 cells were added to the thymoma cells in the absence (ctrl) or presence of 100 mM solutions of various saccharides. The percentage of cells bound relative to the control was determined. Results are expressed as the mean ± SD of duplicate samples in a representative experiment. gal, galactose; glu, glucose; lac, lactose; tdg, thiodigalactoside.

30 min incubation. The binding reached maximum by 90 min, at which time approximately 60% of the MOLT-4 cells were bound to the thymoma cells. No increase in the percent of MOLT-4 cells bound was observed if the incubation was prolonged beyond 90 min. These binding kinetics were essentially identical to that which we had observed for cultured epithelial cells from normal juvenile thymi [18]. Background binding of the radiolabelled T cells to the plate in the absence of thymoma cells was typically less than 3% of the total cpm added to the assay (data not shown).

The effect of various saccharides on MOLT-4 cell binding is shown in Fig. 3B. While the level of binding of the T cells was not significantly affected by the addition of either glucose or galactose, the disaccharides lactose (Galβ1-4Glc) and thiodigalactoside (Galβ1-S-1βGal) both had a dramatic inhibitory effect, reducing binding by approximately 50%. These results indicate that carbohydrate-mediated adhesion accounts for a significant fraction of the cell-cell binding we have observed in this assay. The inhibitory effects of lactose and thiodigalactoside, compared to the monosaccharide galactose, suggested that galectin-1, or a related lectin, participated in the binding, as β-galactosides are preferred oligosaccharide ligands for galectin-1 [16, 17, 20, 21, 28].

Discussion

The present study demonstrates that galectin-1 is expressed in hyperplastic and neoplastic thymi of adult patients with myasthenia gravis. While galectin-1 has been identified in normal juvenile thymus [18], we now document galectin-1 expression in pathologic specimens of human thymus. Our laboratory has recently demonstrated that galectin-1 mediates the interaction of immature T cells to thymic epithelial cells [18]. Thymocytes undergo a number of alterations in cell surface glycosylation as the cells mature into functioning T cells [18, 27, 29]. We have found that galectin-1 preferentially recognizes specific thymocyte cell surface glycoconjugates expressed on immature ($CD3^-$ and $CD3^{lo}$) thymocytes [18]. In the present study, we have demonstrated that MOLT-4 cells, which have a cell surface glycosylation pattern similar to that of immature cortical thymocytes [18, 27], bound to thymoma cells from myasthenic thymi in a carbohydrate-dependent manner (Fig. 3). In this regard, several groups have found that thymocytes present in thymomas with a high lymphocyte to epithelial cell ratio have the cell surface phenotype of immature cortical cells [10, 30]. These findings suggest that expression of galectin-1 by the epithelial component of the thymus in myasthenia gravis may result in the retention or expansion of thymocyte precursors in an organ which typically atrophies during adolescence, giving rise to the abnormal thymus morphology seen in adult patients with this disease. Our observation that a mixture of the inflammatory cytokines IL-1β, TNFα and INFγ with lipopolysaccharide, but not LPS alone, can up-regulate galectin-1 mRNA and protein synthesis ([19], L. Baum, unpublished data) suggests that inflammatory cytokines which circulate in autoimmune disease may increase expression of galectin-1 in the adult thymus. In addition to increased galectin-1 expression, thymomas have also been reported to have increased expression of integrin adhesion molecules [31] which may also contribute to the infiltration of the tumour by thymocytes.

As described above, pharmacologic doses of galectin-1 have been shown to be immunosuppressive in an animal model of myasthenia gravis [22]. The therapeutic effect of exogenous galectin-1 in the animal model may be due to binding and mislocalization of T cells, which play an important role in the pathogenesis of myasthenia gravis [2, 32]. It is not clear whether endogenous galectin-1 expressed in the thymus in myasthenia gravis has an effect on mature, circulating T cells. However, the thymus does appear to have a role in the pathogenesis of myasthenia in humans, since thymectomy is often palliative [7]. This raises the possibility that modulation of galectin-1 mediated cell interactions in the thymus may be a potentially useful therapeutic approach in myasthenic gravis.

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