

# Human Thymic Epithelial Cells Express an Endogenous Lectin, Galectin-1, which Binds to Core 2 O-Glycans on Thymocytes and T Lymphoblastoid Cells

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## Summary

Thymic epithelial cells play a crucial role in the selection of developing thymocytes. Thymocyte–epithelial cell interactions involve a number of adhesion molecules, including members of the integrin and immunoglobulin superfamilies. We found that human thymic epithelial cells synthesize an endogenous lectin, galectin-1, which binds to oligosaccharide ligands on the surface of thymocytes and T lymphoblastoid cells. Binding of T lymphoblastoid cells to thymic epithelial cells was inhibited by antibody to galectin-1 on the epithelial cells, and by two antibodies, T305 and 2B11, that recognize carbohydrate epitopes on the T cell surface glycoproteins CD43 and CD45, respectively. T lymphoblastoid cells and thymocytes bound recombinant galectin-1, as demonstrated by flow cytometric analysis, and lectin binding was completely inhibited in the presence of lactose. The degree of galectin-1 binding to thymocytes correlated with the maturation stage of the cells, as immature thymocytes bound more galectin-1 than did mature thymocytes. Preferential binding of galectin-1 to immature thymocytes may result from regulated expression of preferred oligosaccharide ligands on those cells, since we found that the epitope recognized by the T305 antibody, the core 2 O-glycan structure on CD43, was expressed on cortical, but not medullary cells. The level of expression of the UDP-GlcNAc:Gal $\beta$ 1,3GalNAc-R  $\beta$ 1, 6GlcNAc transferase (core 2  $\beta$ 1, 6 GlcNAc transferase, or C2GnT), which creates the core 2 O-glycan structure, correlated with the glycosylation change between cortical and medullary cells. Expression of mRNA encoding the C2GnT was high in subcapsular and cortical thymocytes and low in medullary thymocytes, as demonstrated by *in situ* hybridization. These results suggest that galectin-1 participates in thymocyte–thymic epithelial cell interactions, and that this interaction may be regulated by expression of relevant oligosaccharide ligands on the thymocyte cell surface.

Interaction between developing thymocytes and thymic epithelial cells is an important step in the maturation of immunocompetent T cells (reviewed in references 1 and 2). Thymic epithelial cells have been proposed to mediate positive selection of thymocytes bearing the appropriate T cell receptor and may also participate in negative selection, or the induction of tolerance (3, 4). Several cell adhesion molecules on thymocytes and thymic epithelial cells have been identified, and some of these have been shown to participate in the binding of human thymocytes to epithelial cells *in vitro*, including the receptor–ligand pairs CD2 and LFA-3, and LFA-1 and intercellular adhesion molecule (ICAM) 1 (1, 2). Other adhesion molecules which have been identified on human

thymic epithelial cells include CD40, CD23, and  $\beta$ <sub>1</sub> integrins (5–8). It has been proposed that different adhesion systems may be used at different points in thymocyte maturation, or by cells in different pathways.

In addition to the protein–protein interactions that mediate cell adhesion, a number of investigators have suggested that carbohydrate–protein interactions may also be involved in the adhesion of thymocytes to thymic epithelium. An endogenous lectin, or carbohydrate binding protein, has been identified in extracts of mouse and chicken thymic stroma (9–11). This lectin was identified as galectin-1 (previously referred to as L14-I, galaptin, and IML-1), a member of the family of S-type, cation-independent lectins (12–15). The galectins

have a common carbohydrate binding domain that preferentially recognizes galactose as a minimal binding determinant, and have been identified in many species, including invertebrates (15). Galectin-1 is a homodimer of 14 kD subunits. The precise function of galectin-1 is not known, although it has been shown to mediate cell-cell (16) and cell-substrate (17, 18) adhesion, apparently by bridging glycoconjugate molecules on opposing surfaces. A role for the murine thymic galectin-1 in thymocyte interactions was suggested by the finding that extracts of murine thymic stroma containing galectin-1 were shown to agglutinate immature, but not mature, murine thymocytes (9). This observation suggested that specific oligosaccharide sequences on immature thymocytes might be ligands for galectin-1 synthesized by thymic stromal cells. In this regard, we have recently demonstrated that expression of a glycosyltransferase enzyme is regulated during thymocyte maturation (19), suggesting that specific thymocyte cell surface oligosaccharides have unique functions at different points in thymocyte development.

We wished to determine whether galectin-1 was present in human thymus, and to examine the physiologic role of galectin in cell-cell interactions in the thymus. We have detected galectin-1 mRNA and protein in human thymic epithelial cells. Galectin-1 appeared to mediate the adhesion of immature T lymphoblastoid cells to thymic epithelial cells, by binding to oligosaccharide ligands on the T cells. Antibody inhibition studies indicated that some of these ligands are O-glycans on specific T cell glycoproteins, the presence of which we have shown is developmentally regulated. These results indicate that carbohydrate-mediated adhesion may participate in the cell-cell interactions that mediate thymocyte maturation. Furthermore, these data suggest that these interactions are regulated by developmentally restricted expression of oligosaccharide ligands on thymocytes.

## Materials and Methods

**Antibodies and Reagents.** The T305 antibody (20) was the kind gift of Dr. Robert Fox (Scripps Clinic and Research Foundation, La Jolla, CA). Murine mAbs to cytokeratin, CD43 (DF-T1), CD45-T (2B11), CD45RO (UCHL1), CD45RA (4KB5), CD45RB (PD7/26), and CD3 (UCHT-1) were obtained from Dako Corp. (Carpinteria, CA). The murine mAb MHM4 (LFA-1 $\alpha$ , CD11a) was obtained from Amac Inc. (Westbrook, ME). The anti-galectin-1 polyclonal rabbit antiserum was obtained as described in (21). Galectin-1 cDNA and purified, recombinant galectin-1 expressed in *Escherichia coli* were obtained as described in (21). Biotinylated galectin-1 for flow cytometry experiments was prepared by coupling *N*-hydroxysuccinimide-biotin (Bethesda Research Laboratories, Gaithersburg, MD) to recombinant galectin-1, according to the manufacturer's instructions. Mono- and disaccharides, *E. coli*  $\alpha$ -D-galactosidase and *Vibrio cholerae* neuraminidase were purchased from Sigma Chemical Co. (St. Louis, MO). The *Pasturella haemolytica* O-sialoglycoprotease was the kind gift of Dr. Alan Mellors (University of Guelph, Canada). [<sup>3</sup>H]Thymidine was purchased from Amersham Corp. (Arlington Heights, IL).

**Cells and Tissue Samples.** Normal human thymus specimens were obtained from infants and children undergoing corrective cardiac surgery at UCLA Hospital. To obtain thymocytes, the tissue was minced, and the cells were collected and passaged over nylon wool

to remove adherent cells and debris. The single cell suspension was maintained for up to 24 h at 37°C in serum-free medium as previously described (22). Primary cultures of human thymic epithelial cells were grown exactly as described by Singer et al. (23).

**Immunofluorescence and Immunohistochemistry.** Thymus specimens were fixed in 4% paraformaldehyde for 24–72 h at 4°C, and embedded in paraffin. For analysis of galectin-1 expression, 6- $\mu$ m sections were incubated with polyclonal rabbit antiserum to galectin-1, diluted 1:1,000 in 0.01 M sodium phosphate, 0.15 M NaCl, 1% BSA, pH 7.5 (PBA)<sup>1</sup> overnight at 4°C. Control sections were incubated with immune rabbit serum which had been absorbed on galectin-1-Sepharose beads, at a final dilution of 1:1,000 in PBA. The sections were washed three times with PBA, and incubated with a goat anti-rabbit reagent conjugated to horseradish peroxidase (HRP; Bio-Rad Laboratories, Richmond, CA), diluted 1:1,000 in PBA, for 2 h at room temperature. Bound antibody was detected by the addition of the chromogenic substrate AEC (3-amino, 9-ethylcarbazole) (Peroxidase Chromogen kit; Biomed, Foster City, CA), and slides were counterstained with hematoxylin. For immunofluorescence studies, the above protocol was followed, except that the thymus sections were labeled simultaneously with the rabbit anti-galectin-1 antiserum and a murine mAb to cytokeratin. Bound primary antibodies were detected with a goat anti-rabbit reagent conjugated to FITC, diluted 1:100 in PBA, and a goat anti-mouse reagent conjugated to rhodamine (TRITC), diluted 1:100 in PBA (Tago Inc., Burlingame, CA).

For analysis of T305 binding, cryostat sections (6  $\mu$ m) of fresh-frozen human thymus were fixed in -20°C methanol for 5 min, preincubated in PBA for 20 min, and then incubated for 2 h at room temperature with the T305 antibody. After washing with PBA, the sections were incubated in HRP conjugated goat anti-mouse Ig (Bio-Rad Laboratories) diluted 1:1,000 in PBA for 2 h at room temperature. Bound antibody was detected with AEC.

**Western Analysis.** Thymic stroma, obtained after removal of thymocytes, was extracted by homogenization in lysis buffer (10 mM Tris, pH 7.3, 130 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.5% NP-40). Cultured thymic epithelial cells and MOLT-4 cells were lysed by brief vortexing in lysis buffer. Nuclei were removed by centrifugation for 2 min at top speed in a microcentrifuge (model 5415; Eppendorf North America, Inc., Madison, WI). 50  $\mu$ g of protein were loaded on SDS-PAGE gels, along with 4  $\mu$ g of purified recombinant galectin-1, and electrophoresed under reducing conditions. Western blot analysis was performed exactly as previously described (21) using the polyclonal rabbit anti-galectin antiserum diluted 1:1,000 in PBS containing 1% nonfat dry milk.

**Northern Analysis.** Total RNA was prepared from cultured thymic epithelial cells by the guanidinium/thiocyanate method (24). Analysis was performed as previously described (19), using a 311-bp EcoRI restriction fragment of human galectin-1 (21) labeled with  $\alpha$ -[<sup>32</sup>P]dCTP by random priming (Amersham).

**Binding Assays and Inhibition Studies.** 1 d before the binding assay, the cultured thymic epithelial cells were passaged into 24-well plastic dishes (Costar Corp.) at a density of  $5 \times 10^5$  cells/well in the absence of the 3T3 feeder cells. This step was performed to ensure that there would be no contamination of the cells with galectin-3 synthesized by the 3T3 cells (15). MOLT-4 cells were labeled overnight with [<sup>3</sup>H]thymidine (50  $\mu$ Ci/ $2 \times 10^6$  cells) in RPMI (GIBCO BRL, Gaithersburg, MD) containing 10% heat-treated FCS and 10 mM Hepes. After labeling, the cells were washed into

<sup>1</sup> Abbreviations used in this paper: HRP, horseradish peroxidase; PBA, PBS plus albumin.

binding medium (RPMI containing 10 mM Hepes and 3% BSA), and resuspended at  $10^5$  cells/ml.  $5 \times 10^4$  MOLT-4 cells were added to the wells containing the thymic epithelial cells and were allowed to bind for the indicated time at either 4 or 37°C. The nonadherent cells were removed by gentle pipetting (sample A). The wells were washed three times with binding medium, and the washes were collected (sample B). The adherent cells were solubilized by the addition of 1 ml of 0.5 N NaOH (sample C). The nonadherent cells, the washes, and the adherent cells were mixed with 1 ml of Ecoscint (National Diagnostics, Inc., Atlanta, GA) and counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The percentage of counts per minute bound was calculated as counts per minute (sample C)/[cpm (sample A) + cpm (sample B) + cpm (sample C)].

In the saccharide inhibition studies, the thymic epithelial cells were preincubated for 30 min with 500  $\mu$ l of a 20 mM solution of the indicated sugar in binding medium. The labeled MOLT-4 cells were added in 500  $\mu$ l of binding medium, so that the final saccharide concentration was 100 mM. For the enzyme treatment studies,  $10^6$  labeled MOLT-4 cells were incubated with 25 mU of *V. cholerae* neuraminidase alone or in combination with 9 mU of *E. coli* exo- $\beta$ -galactosidase in HBSS for 2 h at 37°C. Alternatively,  $10^6$  MOLT-4 cells were incubated with 800  $\mu$ g of *P. haemolytica* O-sialoglycoprotease for 30 min at 37°C. After enzyme treatment, the cells were washed into binding medium, and used in the binding assay.

For antibody inhibition studies, the labeled MOLT-4 cells were resuspended in binding medium containing the indicated antibody immediately before adding the MOLT-4 cells to the thymic epithelial cells. mAbs were used at a final concentration of 5  $\mu$ g/ml. The rabbit antiserum to galectin-1 was passaged over a protein A-Sepharose column to obtain the Ig fraction. This preparation, anti-galectin-1 Ig, was used at a final concentration of 100  $\mu$ g/ml. Control samples contained irrelevant isotype-matched murine mAbs or preimmune rabbit antiserum.

**Flow Cytometry.**  $5 \times 10^5$  cells were incubated with biotin-galectin-1 (50  $\mu$ g/100  $\mu$ l) for 1 h at 4°C in PBA. Cells were washed twice with PBA before staining with a 1:50 dilution of streptavidin-FITC (Boehringer-Mannheim, Indianapolis, IN) or streptavidin-PE (Molecular Probes, Inc., Eugene, OR). After incubation for 1 h at 4°C, the cells were washed twice and resuspended in 500  $\mu$ l PBA. Flow cytometric analysis was performed within 1 h on a FACScan® (Becton Dickinson & Co., Mountain View, CA). For two-color analysis, CD3 antibody directly conjugated to the dye PerCP (Becton Dickinson & Co.) was added to the cells simultaneously with the streptavidin-PE reagent, and the cells were washed and analyzed as described above.

Thymocytes were suspended at  $5 \times 10^6$  cells/ml in PBA. 100  $\mu$ l of cell suspension was combined with 50  $\mu$ l of the appropriate dilution of DFT1 or T305 and incubated on ice for 60 min. The cells were then incubated with FITC-conjugated goat anti-mouse IgG for a second incubation, and finally with PE-conjugated anti-CD3 or PE-conjugated mouse IgG1 for a third incubation. Control samples, containing isotype-matched irrelevant murine antibodies, were included for each sample. After the final incubation, cells were washed twice with cold PBA and resuspended in PBA containing 1  $\mu$ g/ml 7-amino-actinomycin D (7-AAD; Calbiochem-Novabiochem Corp., La Jolla, CA) to discriminate dead cells. Samples were incubated for 20 min at 4°C, protected from light, before acquisition. Nonviable cells, identified by uptake of 7-AAD, were eliminated from the acquisition by selective gating.

**In Situ Hybridization.** Full-length digoxigenin-labeled RNA probes were prepared from pBluescript II KS- (Stratagene, La Jolla,

CA) containing full-length human C2GnT cDNA (25), as previously described (19). The C2GnT was transcribed in the antisense orientation, cut with BamHI, from the T3 promoter, and in the sense orientation, cut with XhoI, from the T7 promoter. Sections of thymus (6  $\mu$ m) on acetylated glass slides were treated with proteinase K and hybridized with the digoxigenin-labeled probes exactly as previously described (19). Hybridization was detected by an anti-digoxigenin/alkaline phosphatase conjugate, followed by 12-h development with the chromogenic substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate.

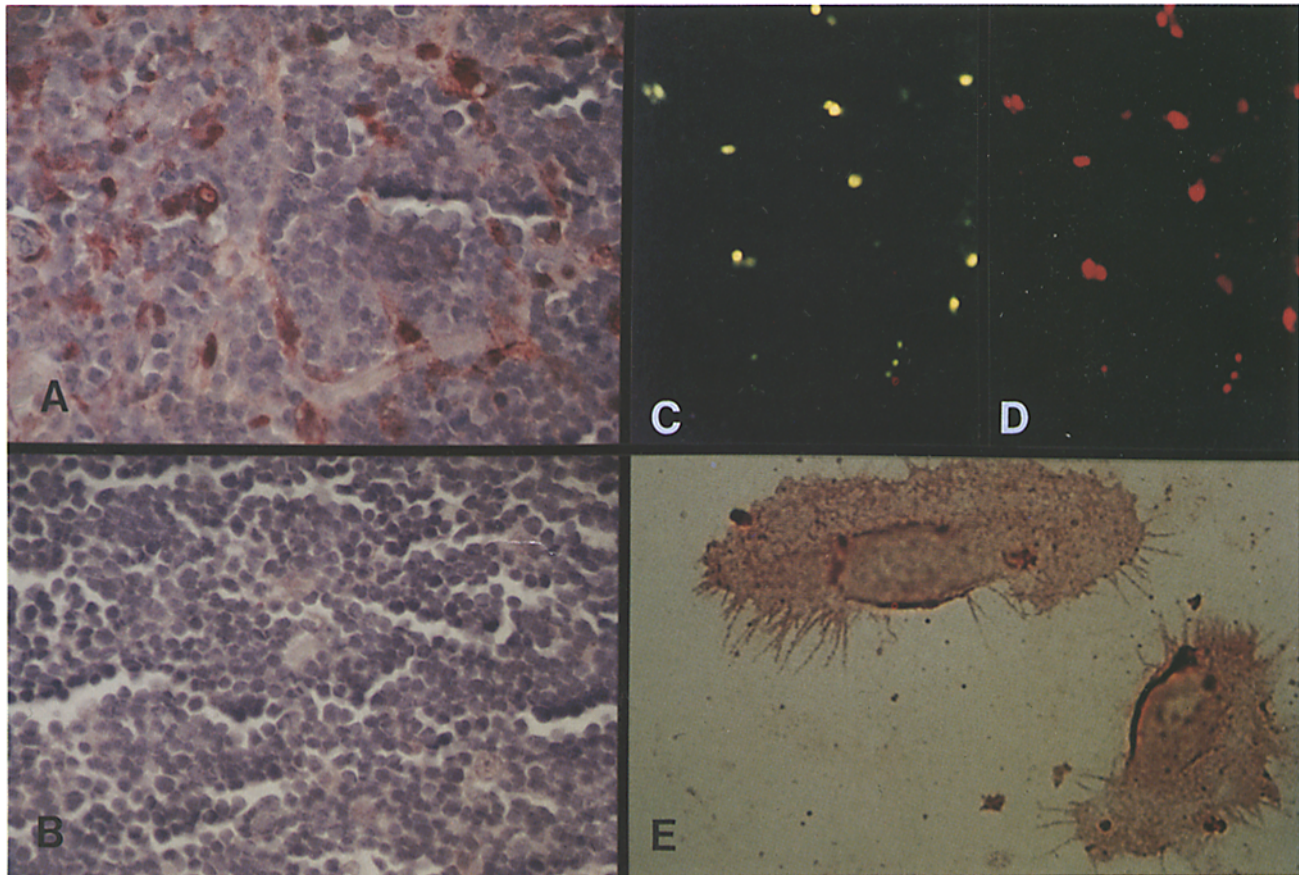
## Results

**Human Thymic Epithelial Cells Express Galectin-1.** To determine whether galectin-1 was present in human thymus, we used a polyclonal rabbit antiserum raised against recombinant galectin-1 for histochemical analysis of human thymus (Fig. 1 A). Individual reactive cells in the thymus sections, which appeared to be large stromal cells, were scattered among the nonreactive thymocytes. The reticular staining pattern seen in Fig. 1 A suggested that the antibody was reacting with antigen on stromal cell processes interdigitating among the thymocytes. The reactive cells were present in both the cortical and medullary zones of the thymus. Antiserum which had been preadsorbed on galectin-1 showed no reactivity with any thymic cells (Fig. 1 B).

To identify the thymic stromal cells expressing galectin-1, we performed double-label immunofluorescence studies. Sections were labeled with anti-galectin-1 antiserum, detected with a fluorescein-tagged reagent, and a mAb to cytokeratin, which reacts with epithelial cells, detected with a rhodamine-tagged reagent. Fig. 1, C and D demonstrates that the same population of cells bound both the antibody to cytokeratin and the antibody to galectin-1, indicating that the cells reacting with the galectin-1 antiserum were thymic epithelial cells. Primary cultures of thymic epithelial cells continued to express galectin-1. Fig. 1 E demonstrates cell-surface labeling of cultured epithelial cells with antibody to galectin-1. Diffuse reactivity was seen on the plasma membrane, extending to the tips of the fine processes put out by these cells in culture.

Immunoreactive galectin-1 was identified in extracts of thymic stroma and of cultured thymic epithelial cells (Fig. 2 A). A single 14-kD band was present in lanes containing the extracts, which comigrated with recombinant galectin-1. In the lane containing recombinant galectin-1, a faint band of 28 kD was present, which corresponds to the homodimer of galectin-1; since the subunits self-associate by hydrogen bonding, but not by formation of cysteine bonds (26), the dimeric form persists in reducing gels.

Northern analysis of mRNA isolated from cultured human thymic epithelial cells demonstrated a single band of ~700 bp (Fig. 2 B). This transcript is identical in size to that encoding the placental gelatin-1 (21). No other bands were detected on Northern analysis, indicating that no other related-14 kD lectins, such as galectin-2 (13), were expressed by the thymic epithelial cells. These results demonstrated that human thymic epithelial cells expressed galectin-1 in vivo, and continued to express galectin-1 mRNA and protein in culture.

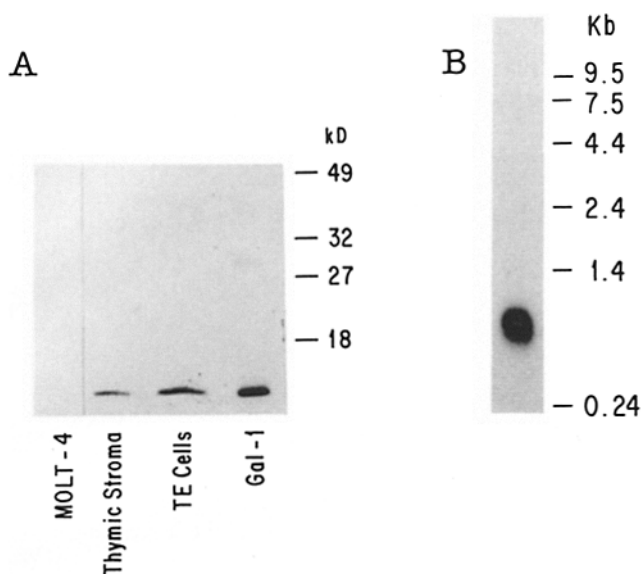


**Figure 1.** Expression of galectin-1 by human thymic epithelial cells. (A and B) Sections of human thymus were incubated with either rabbit antiserum to galectin-1 (A) or with rabbit antiserum to galectin-1 that had been preadsorbed on galectin-1-Sepharose beads (B). Bound antibody was detected by the addition of a goat anti-rabbit Ig reagent conjugated to HRP, which yields a brown reaction product. The slides were counterstained with hematoxylin. (C and D) Sections of human thymus were incubated with either a murine mAb to cytokeratin (C) to label epithelial cells, or rabbit anti-galectin-1 antiserum (D). Bound antibody was detected with either goat anti-mouse Ig conjugated to FITC (C) or goat anti-rabbit Ig conjugated to TRITC (D). Coincident staining patterns were observed, indicating that the cells which expressed galectin-1 were epithelial cells. (E) Primary cultures of human thymic epithelial cells continued to express galectin-1. Nonpermeabilized cells grown on cover slips were incubated with rabbit antiserum to galectin-1, and bound antibody was detected with goat anti-rabbit Ig conjugated to HRP.

*T Lymphoblastoid Cells Bind to Thymic Epithelial Cells in a Carbohydrate-dependent Manner.* To examine the role of galectin-1 in the interaction of thymic epithelial cells with developing T cells, we established a binding assay which measured the binding of a T lymphoblastoid cell line MOLT-4, labeled with [<sup>3</sup>H]thymidine, to cultured thymic epithelial cells (Fig. 3). Labeled MOLT-4 cells were incubated with the adherent thymic epithelial cells for increasing periods of time at 37°C and 4°C. As shown in Fig. 3, ~75% of the MOLT-4 cells bound to the epithelial cells at 37°C; binding was also observed at 4°C, although a smaller percentage of the cells bound at this temperature. At both temperatures, half-maximal binding occurred by 30 min of incubation, and binding reached maximum by 60–90 min of incubation. In contrast, only 11% of labeled BJAB cells, a B lymphoblastoid cell line, were bound to the epithelial cells by 90 min at 37°C. The addition of EGTA (2 mM) to the binding assay had no inhibitory effect on binding, indicating that divalent cations were not required for binding (data not shown).

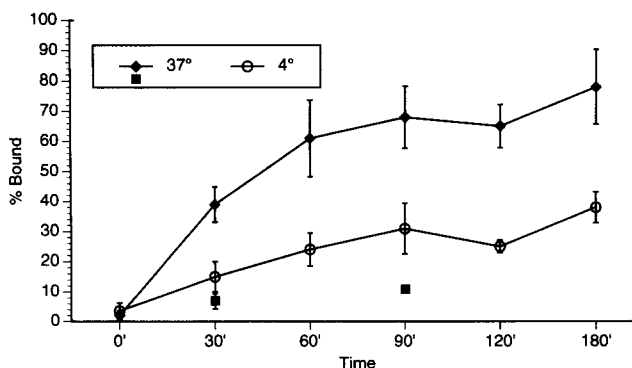
The binding of MOLT-4 cells to the thymic epithelial cells was inhibited by  $\beta$ -galactosides, but not by other simple sugars (Fig. 4). The addition of lactose and thiodigalactoside inhibited binding by 69 and 49%, respectively. The inhibitory effect of the  $\beta$ -galactosides was detectable at concentrations as low as 25 mM (data not shown). No significant inhibition was seen with the other saccharides tested. The inhibitory effects of the  $\beta$ -galactosides, as well as the absence of inhibition by EGTA, suggested that the carbohydrate-mediated adhesion which we observed was mediated by a galectin.

To characterize the T cell surface oligosaccharide ligands involved in binding to thymic epithelial cells, the labeled MOLT-4 cells were treated with a panel of glycosidase enzymes before binding (Fig. 5). Treatment of the cells with *V. cholerae* neuraminidase resulted in an increase in binding, suggesting that sialic acid residues on T cell surface oligosaccharides might be masking ligands that could bind to the epithelial cells. Alternatively, removal of negatively charged sialic acid residues from the T cell surface may have poten-



**Figure 2.** Immunoreactive material in human thymic epithelial cells is galectin-1. (A) Extracts of human thymic stroma, from which the thymocytes were expressed, and of cultured thymic epithelial cells were analyzed by Western blotting for expression of galectin-1. In these samples, a band of 14 kD is seen that comigrates with recombinant galectin-1 (*Gal-1*). MOLT-4 cells, the cells used in binding assays described below, did not express galectin-1. (B) PolyA<sup>+</sup> RNA was prepared from cultured human thymic epithelial cells and was subjected to Northern analysis using a fragment of galectin-1 cloned from human placenta (27) as a probe. A single band of ~ 700 bp was detected.

tiated binding by reducing charge repulsion between T cells and thymic epithelial cells. In contrast, when the cells were incubated with a combination of neuraminidase and *E. coli* exo- $\beta$ -galactosidase, to remove terminal galactose residues, binding was significantly decreased, to ~34% of control. These results indicated that galactose residues on T cell surface glycoproteins participated in the binding of the MOLT-4 cells to the thymic epithelial cells.



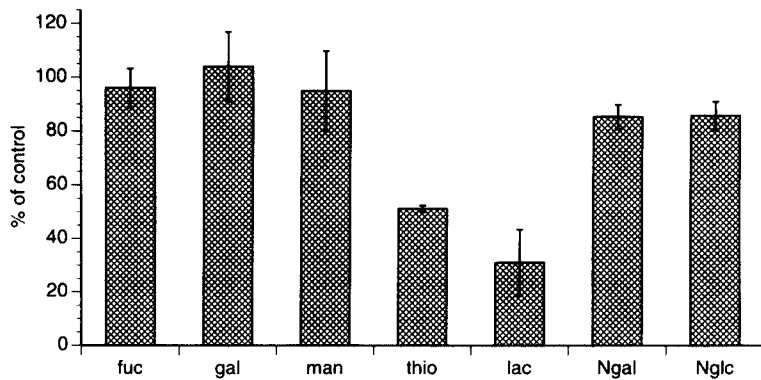
**Figure 3.** [<sup>3</sup>H]Thymidine-labeled MOLT-4 cells bind to cultured human thymic epithelial cells. Labeled cells were incubated with epithelial cells (ratio 1:10) for the indicated times at either 4°C (open circles) or 37°C (closed diamonds). As a control, the binding of the B cell line BJAB at 37°C is shown (closed square). The percentage of added cpm bound was determined. The data are expressed as the mean of three separate experiments, each performed in duplicate,  $\pm$  SEM.

To begin to characterize the oligosaccharide structures on the T cells that were participating in binding, the labeled MOLT-4 cells were treated with *P. haemolytica* O-sialoglycoprotease. This enzyme has been shown to be effective in removing a variety of O-linked oligosaccharide epitopes from T cell surface antigens (27). Treatment of the MOLT-4 cells with the O-sialoglycoprotease reduced binding by ~50% (Fig. 5). These data indicated that T cell surface glycoproteins bearing O-linked oligosaccharides were at least a subset of the ligands involved in carbohydrate-mediated binding of the T cells to thymic epithelial cells.

**Antibody Inhibition Studies Indicate that Two Major T Cell Glycoproteins Are Ligands for Galectin-1.** Figs. 4 and 5 demonstrated that the carbohydrate-mediated binding of MOLT-4 cells to thymic epithelial cells involved galactose residues on the T cells and was inhibitable by  $\beta$ -galactosides, suggesting that a galectin was involved in binding. We examined the ability of anti-galectin-1 Ig to inhibit the binding of MOLT-4 cells to thymic epithelial cells. Addition of anti-galectin-1 Ig to the binding assay inhibited the binding of MOLT-4 cells to thymic epithelial cells by ~50%, compared with control samples containing preimmune rabbit Ig (Fig. 6). Since we did not detect galectin-1 in MOLT-4 cells by Western blot analysis (see Fig. 1), these results suggested that galectin-1 on thymic epithelial cells bound to oligosaccharides on the surface of MOLT-4 cells.

To identify the T lymphoblastoid cell surface glycoproteins that bound to galectin-1 on epithelial cells, we proposed that two T cell surface glycoproteins, CD43 and CD45, might be candidate ligands for galectin-1. Whereas the minimal oligosaccharide recognition structure recognized by galectin-1 may be present on a number of T cell surface glycoproteins, it is likely that only a small number of specific glycoproteins are the biologically relevant ligands for galectin-1 in the thymus, as has been shown for the selectins (28). The large body of data describing structural features of T cell surface antigens indicated that CD43 (leukosialin) and CD45 (common leukocyte antigen) have oligosaccharide sequences that could be ligands for galectin-1. Both of these molecules have large extracellular domains that are heavily O-glycosylated (29), and the enzyme treatments described in Fig. 5 indicated that O-glycans might be recognized by galectin-1 on thymic epithelial cells. In addition, carbohydrate analyses of CD43 and CD45 have demonstrated that both of these molecules can bear poly N-acetylglucosamine sequences, the oligosaccharide sequence preferentially recognized by galectin-1, on O-linked oligosaccharides (28, 30, 31).

mAbs against both CD43 and CD45 inhibited the binding of MOLT-4 cells to thymic epithelial cells (Fig. 6). We first observed that a mAb cocktail which recognizes all isoforms of CD45 inhibited binding by ~44% (CD45, Fig. 6). This reagent is composed of two mAbs, PD7/26 and 2B11. When we assayed these antibodies separately for the ability to inhibit binding, only the 2B11 antibody, which recognizes a T cell-specific isoform of CD45 (32), inhibited the binding of MOLT-4 cells. Antibody to CD45RB alone (PD7/26), as well as other antibodies against the CD45 isoforms CD45RO and CD45RA, had no significant effect on MOLT-4



**Figure 4.**  $\beta$ -galactosides inhibit the binding of MOLT-4 cells to thymic epithelial cells. [ $^3$ H]Labeled MOLT-4 cells were incubated with epithelial cells for 90 min at 37°C, in the presence of the indicated saccharides at a final concentration of 100 mM. The added saccharides were fucose (*fuc*), galactose (*gal*), mannose (*man*), thiodigalactoside (*thio*), lactose (*lac*), N-acetylgalactosamine (*Ngal*), and N-acetylglucosamine (*Nglc*). The bound cpm was determined for each sample, and binding was calculated as the percentage of the cpm bound in the control samples containing no saccharides. The data are expressed as the mean of two separate experiments, each performed in duplicate,  $\pm$  SEM.

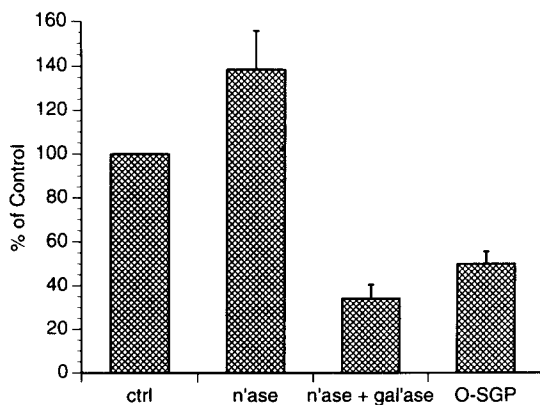
binding. Although the precise structure of the epitope recognized by the 2B11 antibody is not known, these results suggested that the specific epitope recognized by 2B11 participates in the binding of the MOLT-4 cells to the epithelial cells. The inhibitory effect of the 2B11 antibody appeared to be augmented by the addition of the PD7/26 antibody, which may have contributed to blocking the determinant bound by the epithelial cells.

The T305 antibody to CD43 also inhibited binding of MOLT-4 cells by  $\sim$ 45%. The T305 antibody (20) is known to recognize core 2 O-glycans on CD43 (25). Another CD43 antibody, DF-T1, which recognizes CD43 on all thymocytes and T cells, had a more modest inhibitory effect, reducing binding by  $\sim$ 28% (CD43, Fig. 6). The inhibitory effect of the T305 antibody suggested that core 2 O-glycans on CD43 participated in the binding of MOLT-4 cells to thymic epithelial cells.

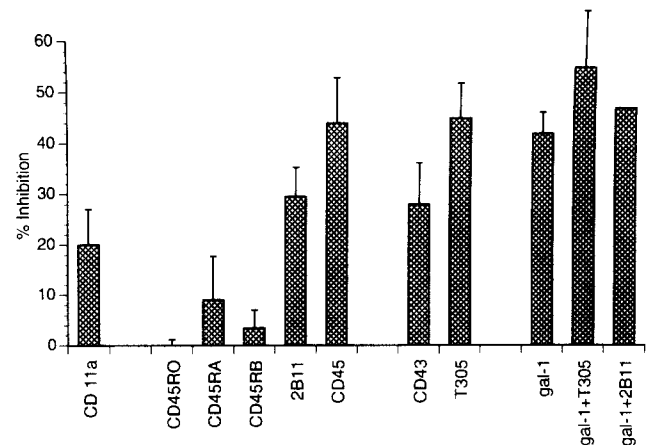
Combining the rabbit antibody to galectin-1 with either

the T305 or 2B11 antibodies again resulted in  $\sim$ 50% inhibition of binding. Since no additive effect over the inhibition observed with the anti-galectin-1 Ig alone was detected, the inhibitory effect of the T305 and 2B11 antibodies on the binding of MOLT-4 cells to thymic epithelial cells was most likely due to disruption of the binding of CD43 and CD45 on the T cells to galectin-1 on the epithelial cells.

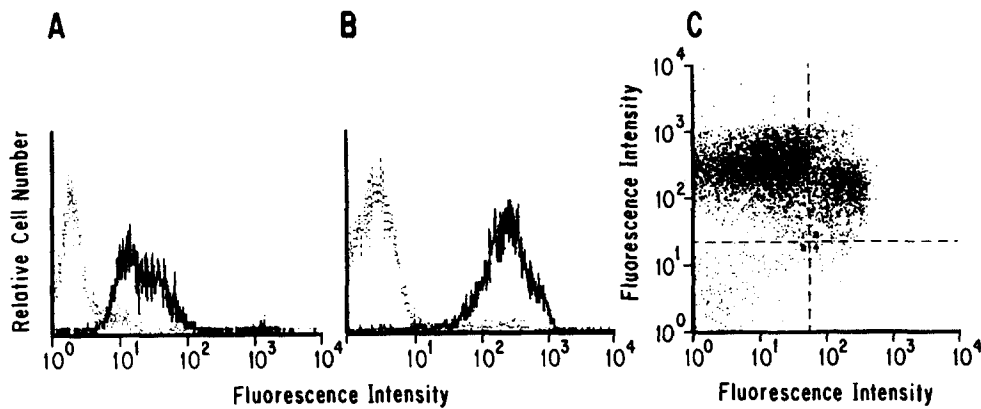
**Galectin-1 Binds to Human Thymocytes.** Our evidence indicated that galectin-1 on thymic epithelial cells bound to cell surface oligosaccharides on MOLT-4 T lymphoblastoid cells. To directly examine the ability of thymocyte and T lymphoblastoid cells to bind galectin-1, we developed a flow cytometry assay in which cells were incubated with biotinylated recombinant galectin-1, and bound lectin was detected with streptavidin-FITC. Fig. 7 A demonstrates that the MOLT-4 cells uniformly bound high levels of galectin-1.



**Figure 5.** The effects of glycosidase treatment on the binding of MOLT-4 cells to thymic epithelial cells. [ $^3$ H]Labeled MOLT-4 cells were treated with *V. cholerae* neuraminidase alone (*n'ase*), with a combination of *V. cholerae* neuraminidase and *E. coli*  $\beta$ -galactosidase (*n'ase* + *gal'ase*) or with *P. haemolytica* O-sialoglycoprotease (O-SGP), or were mock-treated by incubation in buffer alone. The cells were added to the thymic epithelial cells for 30 min at 4°C, and the percentage of cpm bound of the treated cells was compared with that of the control, mock-treated cells. The data are expressed as the mean of three separate experiments, each performed in duplicate,  $\pm$  SEM.



**Figure 6.** Ability of antibodies to galectin-1 and T cell surface glycoproteins to inhibit binding of MOLT-4 cells to thymic epithelial cells. mAbs to LFA-1, CD43, and CD45, (final concentration, 5  $\mu$ g/ml), or protein A affinity-purified rabbit antibody to galectin-1 (*gal-1*) (final concentration, 100  $\mu$ g/ml) were added to the MOLT-4 cells immediately before addition of the cells to the thymic epithelial cells. Binding was performed for 90 min at 4°C. The percentage of cpm bound of the antibody-treated cells was compared with that of control samples. The data are expressed as the mean of two separate experiments, each performed in duplicate,  $\pm$  SEM, except for the *gal-1* antibody and T305 antibody samples, which are the mean of three separate experiments.



**Figure 7.** MOLT-4 cells and human thymocytes bind galectin-1. Cells were labeled with biotinylated galectin-1, followed by either streptavidin-FITC (A) or streptavidin-PE (B and C), and analyzed by flow cytometry. (A) Single-color analysis of MOLT-4 cells labeled with biotinylated galectin-1 (solid line) or biotinylated BSA as a control (broken line). (B) Single-color analysis of freshly isolated human thymocytes labeled with biotinylated galectin-1 (solid line) or biotinylated BSA as a control (broken line). (C) Two-color analysis of freshly isolated human thymocytes

labeled with biotinylated galectin-1 and a murine mAb to CD3, directly conjugated to PerCP<sup>®</sup>. Gates were set using biotinylated BSA and streptavidin-PE on the y-axis, and an isotype-matched murine antibody conjugated to PerCP<sup>®</sup> on the x-axis. The experiment shown is representative of five separate experiments using different thymus specimens.

Binding was completely inhibited when the galectin-1 was preincubated with lactose before addition to the MOLT-4.

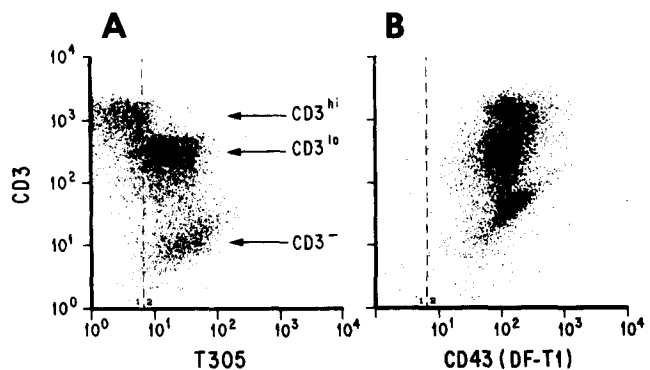
Using this flow cytometry assay, we found that human thymocytes also bound galectin-1. When unseparated thymocytes were analyzed using this method, the majority of the cells bound high levels of galectin-1, whereas a small population of cells bound lower levels of galectin-1 (Fig. 7 B), suggesting that the availability of galectin-1 ligands might differ among thymocyte subpopulations. To relate the degree of galectin-1 binding to the stage of thymocyte maturation, we performed dual-color flow cytometric analysis, using biotinylated galectin-1 and PerCP<sup>™</sup>-conjugated CD3. Fig. 7 C demonstrates that immature CD3<sup>-</sup> and CD3<sup>dim</sup> cells uniformly bound high levels of galectin-1, (relative linear fluorescence intensity = 337 [33]). In contrast, the mature CD3<sup>bright</sup> cells bound slightly less galectin-1, (relative linear fluorescence intensity = 217). The difference in binding did not appear to be due to occupation of binding sites by endogenous galectin-1 which remained bound to the thymocytes during the cell purification and binding assay, since we did not detect any endogenous galectin-1 bound to the cells by flow cytometric analysis using rabbit antiserum to galectin-1 (Wu, T., and L. G. Baum, unpublished data). These results demonstrated that thymocytes expressed cell surface oligosaccharide ligands for galectin-1. These results also indicated that the ability of thymocytes to bind galectin-1 decreased slightly as the cells acquired a mature T cell phenotype.

*CD3<sup>-</sup> and CD3<sup>dim</sup> Thymocytes Express the T305 Antigen.* Our antibody inhibition studies indicated that some of the oligosaccharide ligands on the MOLT-4 cells which appeared to bind to galectin-1 were core 2 O-glycans on CD43, recognized by the T305 antibody. To identify the subsets of thymocytes that express the T305 antigen, we performed two-color flow cytometric analysis using T305 and CD3 mAbs.

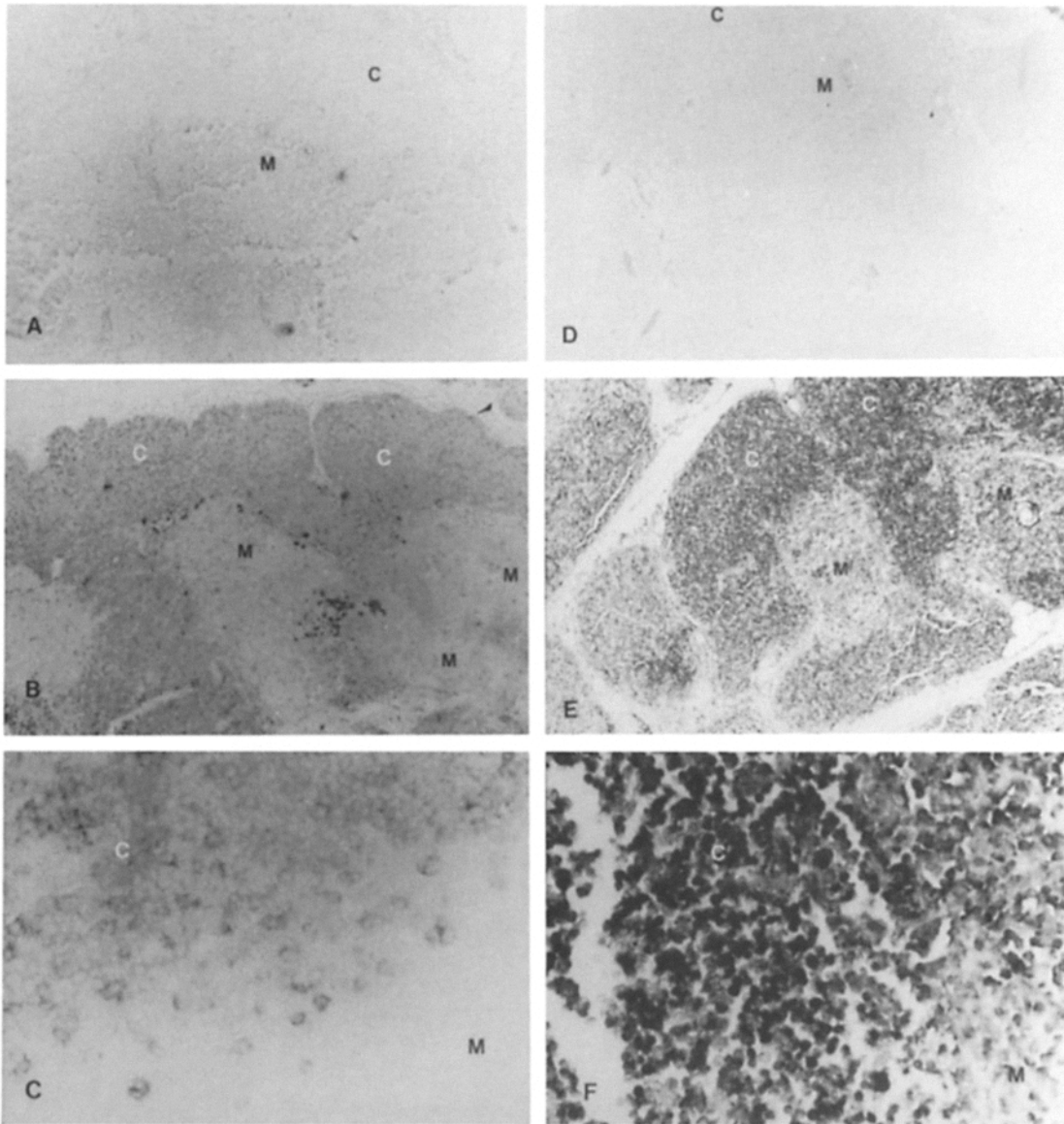
CD3<sup>-</sup> and CD3<sup>dim</sup> thymocytes bound high levels of the T305 antibody (Fig. 8 A). In contrast, the CD3<sup>bright</sup> cells demonstrated no binding of T305 above background levels. The increased level of binding of the T305 antibody by the CD3<sup>-</sup> and CD3<sup>dim</sup> cells was not due to differences in the

level of expression of the CD43 polypeptide. Two-color analysis of the thymocytes using CD3 and the DFT1 antibody, which recognizes CD43 on all thymocytes and T cells, demonstrated equivalent levels of staining of all three populations (Fig. 8 B). These results indicated that the high level of T305 binding to the immature (CD3<sup>-</sup> and CD3<sup>dim</sup>) thymocytes, but not to the mature (CD3<sup>bright</sup>) thymocytes, was related to the presence or absence of the core 2 O-glycans on CD43, and not to a difference in the level of expression of the CD43 protein backbone.

*The Level of C2GnT Expression Correlates with the Presence of the T305 Antigen in the Thymic Cortex.* We performed immunohistochemical staining of sections of thymus with the T305 antibody (Fig. 9). As predicted from the flow cytometric analysis, cells in the subcapsular cortex and the cortex stained strongly with the T305 antibody (Fig. 9, B and C).



**Figure 8.** Expression of CD43 bearing core 2 O-glycans on populations of freshly isolated human thymocytes. Thymocytes were analyzed by two-color flow cytometry, using either the DFT1 (CD43) antibody or the T305 antibody plus goat anti-mouse-FITC, and CD3-PE. CD3 staining identifies CD3<sup>-</sup>, CD3<sup>dim</sup> (CD3<sup>lo</sup>) and CD3<sup>bright</sup> (CD3<sup>hi</sup>) cells, which correspond to increasingly mature thymocyte subpopulations. (A) Fluorescence intensity (on a log scale) of staining with T305 antibody to core 2 O-glycans on CD43 (horizontal axis) vs. CD3 (vertical axis). The gate was set using FITC-conjugated, isotype-matched control mouse IgG. (B) DFT1 vs. CD3.



**Figure 9.** Comparison of T305 reactivity and C2GnT expression in human thymus. Serial sections of thymus were reacted with either the T305 antibody, detected with goat anti-mouse conjugated to HRP, or with digoxigenin-labeled RNA antisense probe to the C2GnT enzyme, detected with antidigoxigenin antibody conjugated to alkaline phosphatase. The cortical (C) and medullary (M) regions of the thymus are indicated on each panel. (A) Control antibody staining with isotype-matched mouse Ig. (B and C) Staining with the T305 antibody ( $\times 100$  and  $\times 400$ ). (D) Control in situ hybridization with sense orientation C2GnT probe. (E and F) In situ hybridization with antisense orientation C2GnT probe ( $\times 100$  and  $\times 400$ ).

These cells are typically CD3<sup>-</sup> and CD3<sup>dim</sup>, respectively. In contrast, only scattered cells in the medulla, where most thymocytes are CD3<sup>bright</sup>, reacted with the T305 antibody. Fig. 9 C demonstrates that the antibody staining is seen primarily on the cell surface, although intracellular staining is seen in a few cells. The intracellular staining most likely results from the T305 antibody binding to core 2-containing CD43 molecules still in the secretory pathway, which are exposed by tissue sectioning. No specific staining was seen in sections incubated with an isotype-matched control murine mAb (Fig. 9 A).

The presence of the T305 antigen on the cell surface glycoprotein CD43 results from expression of the C2GnT (25),

suggesting that cells which bind T305 may have a higher level of expression of the C2GnT enzyme. To relate the pattern of T305 binding to expression of the C2GnT in human thymus, we performed in situ hybridization on serial sections of thymus tissue, using an antisense RNA probe for the C2GnT. The pattern of expression of the C2GnT in the in situ hybridization analysis correlated exactly with the T305 immunohistochemical staining pattern (Fig. 9 E). Cells in the subcapsular cortex and the cortex demonstrated strong reactivity with the antisense probe, as shown by the darkly stained cells in these regions, whereas most cells in the medulla demonstrated only faint reactivity. Fig. 9 F shows the strong cytoplasmic signal detected in the cortical cells with the C2GnT



probe. Control sections, incubated with a sense orientation probe for the C2GnT, showed no hybridization signal (Fig. 9D). The correlation between T305 antigen expression and C2GnT expression indicates that developmentally regulated expression of the C2GnT enzyme accounts for the presence of core 2 O-glycans on immature thymocytes.

## Discussion

The present study demonstrates that cell surface oligosaccharides on immature T cells mediate adhesion of these cells to thymic epithelium, at least in part via the endogenous epithelial cell lectin galectin-1. Our laboratory and others have documented the complex changes in thymocyte cell surface glycosylation which occur during thymocyte maturation (19, 34–36). The precise regulation of oligosaccharide synthesis during T cell development suggests that different oligosaccharide sequences have unique functions at specific points in thymocyte maturation. However, no study has directly demonstrated a role for thymocyte cell surface oligosaccharides in cell–cell interactions.

Galectins have been proposed to participate in fetal development (37), in the migration of myoblasts on laminin (17, 18), in regulation of cell growth (38–40), and in tumor cell adhesion and metastasis (16, 41). Homodimeric galectin-1 appears to mediate cellular adhesion by binding to glycoprotein ligands on apposing cells or on cells and extracellular matrix components, bridging these structures. In different tissues, specificity of galectin-mediated interactions may result from the selective expression of different glycoprotein ligands in the tissues (42). Our data, demonstrating that galectin-1 on human thymic epithelial cells binds to thymocytes bearing the appropriate oligosaccharide ligands, is the first report of a role for galectin-1 in lymphocyte development.

Fig. 4 demonstrates that  $\beta$ -galactosides inhibited the binding of MOLT-4 cells to thymic epithelium. The requirement for millimolar concentrations of simple sugars for inhibition of binding is a common feature of lectin–ligand interactions in complex biological systems. This reflects the fact that, whereas a simple saccharide structure may be the minimal recognition structure bound by most animal lectins, the physiologic ligands bound by these lectins are more complex, and are typically specific glycoproteins which bear multiple saccharide structures (42).

The T cell surface ligands recognized by galectin-1 include glycoproteins bearing O-glycans, since O-sialoglycoprotease treatment of the T cells significantly reduced binding of the cells to thymic epithelium (Fig. 5). Additional oligosaccharide ligands may be N-glycans, which can also bear the poly-N-acetylglucosamine sequences preferentially bound by S-type lectins (18, 42). The two T cell surface glycoproteins which we identified as candidate ligands for galectin-1, CD43 and CD45, were examined because these are extensively O-glycosylated molecules with mucinlike domains (29). Both of these molecules have been shown to acquire carbohydrate differentiation antigens during thymocyte development (34–36, 43) and poly-N-acetylglucosamine sequences, the oligosaccharide structures preferentially recognized by galectin-1, have

been identified on both CD43 and CD45 (30, 31). Adhesive (44, 45) and antiadhesive (46, 47) properties of CD43 and CD45 have been demonstrated, although the role of the oligosaccharides in these functions is not clear.

Antibodies to galectin-1, as well as specific antibodies to CD43 and CD45, blocked binding of MOLT-4 cells to thymic epithelial cells (Fig. 6). The epitope recognized by the T305 antibody, the core 2 O-glycan on CD43, is created by expression of a specific N-acetylglucosaminyltransferase (core 2 GnT) which initiates formation of the branch to which the lactosamine unit is added (25). Inhibition by the T305 antibody suggested that galectin-1 bound to core 2 O-glycans on CD43, perhaps via the lactosamine sequences on the oligosaccharide chains. In all cases, the maximal level of antibody inhibition that we observed was  $\sim 50\%$ . This level of inhibition of T cell–epithelial cell binding is similar to that reported in other systems, in which antibodies to thymocyte cell surface molecules such as integrins were demonstrated to block binding (48). This may indicate that multiple adhesion systems participate in cell–cell binding in *in vitro* assays. To minimize some of these other interactions, we performed the binding assays at  $4^\circ\text{C}$ , a temperature at which LFA-1/ICAM-1-mediated binding of thymocytes or T lymphoblastoid cells is inhibited *in vitro* (48). As shown in Fig. 6, antibody to the  $\alpha$  subunit of LFA-1 had a minimal inhibitory effect on binding. In addition, the MOLT-4 cell line used for this study does not express cell surface CD2 (Uittenbogaart, C.H., unpublished data), which is known to participate in thymocyte adhesion (1).

Antibodies to CD43 have been shown to cause shedding of the molecule from the cell surface (49). We examined whether binding of galectin-1 would mimic this effect, by incubating MOLT-4 cells with galectin-1 and then examining cell surface CD43 expression after dissociation of bound galectin-1 with lactose. We have found no decrease in cell surface levels of CD43 on MOLT-4 cells after binding of galectin-1 (Giese, M., and L.G. Baum, unpublished data).

Flow cytometric analysis of galectin-1 binding to thymocytes demonstrated that thymocytes bound galectin-1, with immature thymocytes binding slightly more galectin-1 than mature thymocytes (Fig. 7). This pattern of binding is similar to the pattern of binding of the T305 antibody to human thymocytes. Figs. 8 and 9 demonstrate that the presence of core 2 O-glycans, detected by the T305 antibody, as well as the expression of the core 2 GnT, are regulated during thymocyte development, with the highest level of expression seen in immature cortical thymocytes. These results suggest that the degree of binding of thymocyte glycoproteins to galectin-1 on epithelial cells may be regulated by controlling the synthesis of the oligosaccharide ligands during thymocyte development. According to this model, regulated expression of glycosyltransferase enzymes, such as the core 2 GnT, would create cell surface oligosaccharides on immature thymocytes which would be recognized by galectin-1 on epithelial cells. Thymocyte maturation would be accompanied by downregulation of the glycosyltransferase enzymes, and *de novo* synthesis of glycoproteins lacking the oligosaccharide recognition structures, resulting in dissociation from galectin-1.

The importance of endogenous lectins in lymphocyte trafficking and cell-cell communication is becoming increasingly apparent. In addition to carbohydrate binding proteins such as the selectins (28) and the B cell antigen CD22 (50), it appears that galectins may also play a role in regulating lymphocyte adhesion. Other studies (51, 52) have demon-

strated that galectins are immunosuppressive, in animal models of autoimmune diseases. Whereas the full role of galectin-1 in modulating immune function is not yet understood, the present study indicates that galectin-1 participates in crucial cell-cell interactions during T cell development.

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