AD_____

Award Number: DAMD17-02-1-0022

TITLE: Elucidation of a Novel Cell Death Mechanism in Prostate Epithelial Cells

PRINCIPAL INVESTIGATOR: Linda G. Baum, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of California
Los Angeles, California 90024-1406

REPORT DATE: December 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2003	Annual (19 Nov 2002 - 18 Nov 2003)

4. TITLE AND SUBTITLE
Elucidation of a Novel Cell Death Mechanism in Prostate Epithelial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0022

6. AUTHOR(S)
Linda G. Baum, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California
Los Angeles, California 90024-1406

E-Mail: lbaum@mednet.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Tumor cell resistance to apoptosis is a major obstacle to effective therapy of prostate cancer. We have found that the androgen dependent prostate cancer cell line LNCaP is sensitive to apoptosis induced by galectin-1, an endogenous human lectin that is abundant in prostate stroma. In contrast, androgen independent LNCaP, DU145, and PC3 cells are resistant to galectin-1 induced death and actually synthesize galectin-1 and export it to the cell surface. Galectin-1 binds to saccharide ligands on suscepibel LNCaP cells to trigger cell death. Susceptibility to galectin-1 appears to depend on the presence of a specific class of cell surface glycans, the O-linked glycans on glycoproteins; in contrast, N-glycans are not required for galectin-1 induced LNcaP cell death. Resistance to galectin-1 induced death correlates with markedly decreased expression of a specific glycosyltransferase, the C2GnT, which creates saccharide ligands on O-glycans that are recognized by galectin-1. The C2GnT enzyme also regulates susceptibility of T cells to galectin-1 induced death, indicating that a common glycosylation pathway may control cell death in epithelial and lymphoid cells. Identification of a mechanism that enhances galectin-1 prostate cancer cell death mal allow novel therapeutic approaches to manipulate tumor cell glycosylation to overcome tumor cell resistance to apoptosis.

14. SUBJECT TERMS
Apoptosis, cell death, galectin-1, glycosylation, sialyltransferase

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Blocking the ability of tumor cells to survive in different anatomic sites and tissues is an important goal in controlling the spread of cancer. We are examining apoptosis, or programmed cell death, induced by a human lectin termed galectin-1. The galectins are a family of mammalian lectins, or carbohydrate binding proteins, that have multiple functions, including positive and negative regulation of cell death. Galectin-1 is abundant in many organs, especially in the prostate stroma. Galectin-1 was initially reported to kill subsets of B and T lymphocytes. However, additional reports have demonstrated that an androgen-dependent prostate cancer cell line undergoes apoptosis after binding soluble galectin-1, while two androgen-independent prostate cancer cell lines are resistant to galectin-1 induced death. Thus, galectin-1 may be a general death trigger for a variety of cell types. As resistance to apoptosis is a hallmark of many types of cancers, we wish to understand the mechanisms governing susceptibility or resistance of various prostate cancer cell lines to galectin-1 induced apoptosis. Understanding the regulation of prostate cancer cell death will allow the development of novel therapeutic approaches to eliminate tumor cells.

Body

The approved Specific Aims and Statement of Work for this project are below. In our second year of funding, we have made substantial progress in Tasks 2, 3, and 4, as described following the Specific Aims and Statement of Work. We have also performed preliminary work essential for the completion of Task 5.

Specific Aims: 1) Characterize features of galectin-1 death in prostate cancer cell lines. 2) Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells, and determine if regulated glycosyltransferase expression controls cell death. 3) Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors. 4) Characterize the cell surface receptors for gal-1 on prostate cancer cells and compare the pattern of expression of these receptors on galectin-1 sensitive and gal-1 resistant prostate cancer cell lines. Determine the requirement for specific cell surface receptors in galectin-1 mediated death of prostate cells. 5) Investigate the intracellular galectin-1 death pathway, and identify associations between prostate-specific cell surface receptors and common intracellular death pathway components.

Statement of Work

Task 1. To characterize features of galectin-1 death in prostate cancer cell lines (months 1-3).
a. Examine hallmarks of cell death, including membrane lipid assymetry, cell permeability, loss of mitochondrial membrane potential and DNA fragmentation. (months 1-3)
b. Examine calcium flux, caspase activation and cytochrome C release, to identify novel features of the galectin-1 death pathway in prostate cancer cells. (months 1-3)

Task 2. To identify the roles of specific glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells (months 1-18).
a. Examine expression patterns of ST6Gal I, ST3Gal I, FucT VII, GnTV and C2GnT glycosyltransferases in galectin-1 sensitive and insensitive LnCaP cell lines (months 1-6).

b. Determine effects of expressing ST3Gal I and FucT VII that mask and C2GnT that create galectin-1 saccharide ligands on prostate cell susceptibility to galectin-1 (months 6-18).
c. Examine prostate cancer biopsies for the pattern of glycosyltransferase expression in primary prostate cancers (months 6-12).

Task 3. To determine whether galectin-3 opposes the effects of galectin-1 in prostate cancer cell lines (months 6-18).
a. Express galectin-3 in a galectin-1 sensitive LnCaP cell line (months 6-9).
b. Determine effect of galectin-3 overexpression on galectin-1 susceptibility (months 9-12).
c. Perform competition experiments with soluble recombinant galectin-1 and galectin-3 (months 12-18).

Task 4. To examine the requirement for specific prostate cell surface glycoprotein receptors for galectin-1 induced death (months 12-36).
a. Following isolation and characterization of receptors, determine receptor expression pattern on galectin-1 resistant cells (months 12-24).
b. Characterize receptor domains that are required for sending the death signal
c. Examine requirements for specific receptors by antibody inhibition, anti-sense transfection or expression of dominant negative receptor constructs in galectin-1 susceptible cells or by expression in galectin-1 resistant cells, (months 24-36).

Task 5. To investigate the intracellular galectin-1 death pathway (months 12-36).
a. Determine effects of protein kinase C and protein phosphatase inhibitors on galectin-1 susceptibility (months 12-18).
b. Identify additional candidate signaling molecules based on identities of cell surface receptors determined in Task 4 (months 18-30).
c. Determine requirement for specific signaling molecules by expression of relevant wildtype or mutant constructs in specific cell lines (months 18-36).
d. Identify association between cell surface receptors and signaling molecules by immunoprecipitation and confocal immunofluorescent microscopy (months 24-36).

Aim 1. Characterize features of galectin-1 death in prostate cancer cell lines

We have used the LNCaP cancer cell line that is susceptible to galectin-1 induced cell death. Last year, we completed **Task 1a**, confirming that LNCaP cells demonstrate many hallmarks of cell death, including DNA fragmentation, uptake of propidium iodide, and plasma membrane lipid asymmetry as demonstrated by annexin V binding.

In year 2, we have concentrated on **Task 1b**, examining intracellular features of the galectin-1 death pathway. Initially, we concentrated on markers that we had previously identified in the T cell death pathway triggered by galectin-1. However, we have made a novel observation regarding a new potential player in the galectin-1 death pathway. In examining various kinase inhibitors for the ability to block galectin-1 induced T cell death, we discovered that a number of serine/threonine kinase inhibitors actually enhanced galectin-1 induced apoptosis. This suggested that antagonizing these kinases, by the action of serine/threonine phosphatases, might be inhibitory for galectin-1 death. We screened a panel of phosphatase inhibitors and found that only those compounds that blocked the PP2A phosphatase blocked galectin-1 induced death (Fig. 1). This was very exciting, as this phosphatase is ubiquitously

expressed in a variety of tissues, a prerequisite for defining a generalizable galectin-1 death pathway. In addition, recent work by Thompson and co-workers has shown that PP2A inhibition via caveolin signaling was important for survival of LNCaP cells (1), suggesting that galectin-1 signaling via PP2A activation will also regulate apoptosis of prostate cancer cells. We are currently testing the same panel of inhibitors shown in Fig. 1 on LNCaP cells, to determine if PP2A inhibitors selectively block galectin-1 induced cell death.

Aim 2. Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death and determine if regulated glycosyltransferase expression controls cell death.

Last year, we completed **Task 2a**, examining expression patterns of a variety of glycosyltransferase enzymes that could create or modify oligosaccharide ligands for galectin-1. We found that O-glycans, but not N-glycans, appeared to regulate susceptibility to galectin-1. Based on that data, we focused on modifying O-glycans in **Task 2b**, as we had identified that LNCaP cells that are susceptible to galectin-1 death express the C2GnT enzyme that modifies O-glycan structures, while galectin-1 resistant cells had markedly reduced expression of the C2GnT. This again was an important observation, as the C2GnT enzyme is important for regulating galectin-1 induced death of T cells; again, the generalizability of the death pathways in T cells and in prostate cancer cells is remarkable, given that prostate cells express none of the polypeptides that bear core 2 O-glycans on T cells.

In year 2, we have made considerable progress in **Task 2b**. We took two approaches, down-regulating the C2GnT and overexpressing the ST3Gal I enzyme that competes with the C2GnT, to block this oligosaccharide modification pathway; the latter approach, overexpressing the ST3Gal I, has been more successful. As shown in Fig. 2, overexpression of the ST3Gal I in LNCaP cells resulted in blocking extension of O-glycans, as shown by loss of PNA staining. Importantly, expression of the ST3Gal I significantly reduced the susceptibility of LNCaP cells to galectin-1, demonstrating that O-glycans are critical ligands in the galectin-1 death pathway in these cells, and that modification or loss of these O-glycan ligands may contribute to apoptosis resistance and tumor progression in prostate cancer.

This work was presented at a Gordon Conference on Glycobiology in March, 2003. Subsequently, Fukuda and co-workers have published an abstract demonstrating that C2GnT expression in prostate cancer biopsies is an independent predictor of cancer progression (2).

In the coming year, we will concentrate on the final task in Aim 2, **Task 2c**, examining prostate cancer biopsies for glycosyltransferase expression and determining if the pattern of glycosyltransferase expression that protects against galectin-1 apoptosis in cell lines is seen in primary cancers.

Aim 3. Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors.

As a first step in Aim 3, we have screened a panel of prostate cancer cell lines for expression of galectin-1 and galectin-3 and susceptibility to galectin-1 induced cell death. As shown in Table 1, this includes cell lines generated in the lab of Dr. Charles Sawyers at UCLA, with whom we have established a collaboration. Notably, the only galectin-3 negative cell line is LNCaP, which is also the only cell line that we have identified to date that is susceptible to galectin-1 induced cell death. This observation supports our hypothesis that, as we have found in

T cells, galectin-3 expression in prostate cancer may protect cell from apoptotic death; this role for galectin-3 has now been established in breast cancer (3).

In collaboration with Fu-Tong Liu (UC Davis) and Mike Teitell (UCLA), we have also demonstrated a role for galectin-3 in lymphoma, specifically resistance to apoptosis in diffuse large B cell lymphoma (4). Importantly, we also found that the C-terminal domain of gal-3 acted as a dominant negative (DN) and abolished galectin-3 mediated apoptosis resistance, consistent with recent work by John and co-workers that the C-terminal domain of galectin-3 inhibited tumor growth and metastasis in breast cancer in a mouse model (5). Thus, in the coming year, we will expand our original Aim 3. First, we will overexpress galectin-3 in LNCaP cells to determine if this blocks galectin-1 mediated cell death (6); this completes **Task 3a** and allows us to quickly complete **Task 3b**. Second, we will express the DN galectin-3 C-terminal domain in these cells and in other cells in Table 1 that express endogenous galectin-3, and determine if this enhances susceptibility of these cells to apoptosis. This may provide a novel approach for overcoming prostate cancer resistance to apoptosis in patients.

For **Task 3c**, we will ask if extracellular galectin-1 and galectin-3 would compete for the same glycoprotein counterreceptors on the cell surface. For these assays, we have produced biotinylated galectin-1 and galectin-3 and developed a binding assay in which one unlabeled galectin is used to compete the binding of the labeled galectin. We have validated this assay with T cell lines (Fig. 3) and are now performing these assays with LNCaP cells.

Aim 4. Characterize the cell surface receptors for galectin-1 on prostate cancer cells

Our initial approach in **Task 4a** was to focus on candidate glycoprotein receptors expressed on prostate cancer cells. As galectin-1 has been shown to bind to the cancer mucin CA-125, and recent data from our lab suggests that some galectins may recognize CD44, we proposed the prostate cancer mucin Muc18 and CD44 as potential receptors involved in galectin-1. However, the galectin-1 susceptible LNCaP cells express neither of these potential receptors. Thus, as an alternative approach, we are now using the lectin PNA to screen for candidate glycoprotein receptors for galectin-1. As described in Aim 2, O-glycans recognized by PNA appear to be important for galectin-1 induced death. We are currently performing lectin blots and lectin precipitations with PNA of both parental LNCaP cells and the LNCaP cells expressing the ST3Gal I, to identify the major O-glycosylated proteins and to determine which of these is modified by ST3Gal I expression, leading to reduced PNA binding (Fig. 2). We will continue with this approach, and move onto **Tasks 4b and 4c** in the coming year.

Importantly, we moved into an exciting and productive new direction this year in examining the functions of tumor cell galectin-1. Work of Castronovo and co-workers had demonstrated abundant deposition of galectin-1 in the stroma of prostate cancers, with increasing galectin-1 deposition positively correlating with disease progression and poor patient outcome (7,8); this group had proposed that galectin-1 acts as an "immunologic shield" around tumors. A post-doctoral fellow in the lab, James He, directly addressed this hypothesis, by growing galectin-1 producing stromal cells on plastic or on the extracellular matrix material Matrigel. Dr. He made several remarkable observations. First, the stromal cells secreted increased amounts of galectin-1 when growing on Matrigel, indicating that the matrix material increased secretion of galectin-1 by mass action removal from the stromal cells. Second, when Lec8 stromal cells that secrete misfolded galectin-1 were grown on Matrigel, the galectin-1 folded properly on the Matrigel, as demonstrated by binding and functional assays. Third, galectin-1 secreted onto the matrix could kill T cells, without any contact with the stromal cells. Fourth, 10-fold less

galectin-1 was required to kill T cells when the galectin-1 was presented by extracellular matrix, compared to soluble galectin-1. Fifth, death of T cells occurred very rapidly, with cells becoming annexin V^+ within 60 minutes of binding to galectin-1 on matrix; this implies that tumors that secrete galectin-1 will thwart any immunotherapy approaches to controlling cancer.

These findings have profound implications for our understanding of tumor defenses against the immune system, and this work is in press (9, Appendix 1). We are now performing these studies with different prostate cancer cell lines that do or do not secrete galectin-1, to demonstrate that galectin-1 resistance by prostate cancer cells may confer a selective advantage *in vivo* by thwarting the immune response to the cancer.

Aim 5. Investigate the intracellular galectin-1 death pathway, and identify associations between prostate-specific cell surface receptors and common intracellular death pathway components

As described on page 3, we have found that PP2A inhibits T cell death induced by galectin-1 (Fig. 1) and are currently examining the effects of PP2A inhibitors on death of LNCaP cells by galectin-1. Additional preliminary data demonstrates that, in T cells, PP2A appears to associate with CD7, a cell surface glycoprotein that is a receptor for galectin-1 (Hernandez and Baum, unpublished data). In the coming year, we will identify PNA binding proteins as described on page 7, and determine by lectin precipitation and Western blotting if PP2A associates with any of these receptors. Our hypothesis is that galectin-1 can kill diverse types of cells by binding common oligosaccharides, e.g. created by the C2GnT, on various polypeptide backbones; these polypeptides, while not identical from cell type to cell type, have common features, so that the intracellular domain of one or more receptors will associate with PP2A and trigger the galectin-1 death pathway. We will pursue this hypothesis as we complete **Task 5** in the coming year.

Key Research Accomplishments

- Identification of protein phosphatase 2A as a possible common mediator of galectin-1 induced cell death.

- Expression of the ST3Gal I in LNCaP cells, and demonstration that the expressed enzyme is functional by reduced PNA binding detected by flow cytometry.

- Demonstration that sialylation of core 1 O-glycans by the ST3Gal I, that would block extension of core 2 O-glycans, reduced LNCaP apoptosis by galectin-1 by approximately 30%.

- Characterization of different prostate cancer cell lines for expression of various galectin family members.

- Determination that the C-terminal domain of galectin-3 acts as a dominant negative in blocking the anti-apoptotic effects of galectin-3.

- Exclusion of Muc18 and CD44 as candidate receptors essential for galectin-1 cell death, and identification of PNA-binding cell surface glycoproteins as potential receptors essential for galectin-1 cell death.

- Demonstration that galectin-1 presented on extracellular matrix can kill T cells, and that galectin-1 presented on matrix kills T cells 10-fold more efficiently than we have observed with soluble galectin-1.

Reportable outcomes

Presentation of this work in two posters, by Dr. James He and Dr. Hector Valenzuela, at the 2003 6th Annual San Diego Glycobiology Symposium.

Presentation of this work by the P.I. in an invited platform presentation at the 2003 Gordon Conference on Glycobiology.

Presentation of this work in a poster and brief platform presentation, by Joseph Hernandez, at the 2003 Gordon Conference on Glycobiology.

Publication of a manuscript describing matrix-associated galectin-1 induction of T cell death.
He J and **Baum LG** (2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. *Journal of Biological Chemistry* (in press)

Conclusions

In the second year of this project, we have continued to make substantial and rapid progress in defining the features and mechanisms of galectin-1 induced death of prostate cancer cells. Importantly, we have proven one of our major hypotheses, i.e. that glycosylation controls prostate cell susceptibility to apoptosis, as we have found that over-expression of the ST3Gal I glycosyltransferase that masks core 1 O-glycans, significantly reduced the susceptibility of LNCaP cells to galectin-1 induced death. Moreover, this work demonstrates that common mechanisms such as glycosylation of cell surface glycoproteins regulate susceptibility to galectin-1 death in different cell types, T cells and prostate cancer cells.

Another important accomplishment is our demonstration that galectin-1 in extracellular matrix kills T cells, thus validating the hypothesis that galectin-1 secreted into prostate cancer can act as an "immunologic shield" protecting the tumor from immune attack.

In our final year of this project, we anticipate that we will continue our focused, rapid progress on this problem, and will submit 1-2 manuscripts on this work.

"So what section": Our work shows that tumors develop molecular mechanisms to protect themselves from cell death. These mechanisms may be common among different types of cells. In addition to escaping cell death, prostate cancer cells that can resist apoptosis induced by galectin-1 can secrete this protein, to kill invading immune cells attempting to attack the tumor. By controlling the mechanisms that regulate prostate cancer cell susceptibility to death, we can exploit two potential pathways to control prostate cancer, i.e. direct killing of the cancer cells by apoptosis inducing agents such as galectin-1, and enhanced immune attack of the tumor by lymphocytes.

References

1. Li L, Ren CH, Tahir SA, Ren C, Thompson TC (2003) Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol. 23:9389-404

2. Ohyama C, Hagisawa S, Hatakeyama S, Arai Y, Fukuda M (2003) Core 2 β1,6-N-acetylglucosaminyltransferase (C2GnT) expression in human prostate cancer: a predictor for non-organ confined disease and biochemical relapse after radical prostatectomy. Glycobiol 13: abstract 143

3. Moon BK, Lee YJ, Battle P, Jessup JM, Raz A, Kim HR (2001) Galectin-3 protects human breast carcinoma cells against nitric oxide-induced apoptosis: implication of galectin-3 function during metastasis. Am J Pathol 159:1055-60

4. Hoyer KK, Pang M, Kuwabara I, Liu F-T, Said JW, Baum LG, Teitell MA (2004) An anti-apoptotic role for galectin-3 in diffuse large B cell lymphomas. Am J Pathol (in press)

5. John CM, Leffler H, Kahl-Knutsson B, Svensson I, Jarvis GA (2003) Truncated galectin-3 inhibits tumor growth and metastasis in orthotopic nude mouse model of human breast cancer. Clin Cancer Res 9:2374-83

6. Ellerhorst JA, Stephens LC, Nguyen T, Xu XC (2002) Effects of galectin-3 expression on growth and tumorigenicity of the prostate cancer cell line LNCaP. Prostate 50:64-70

7. van den Brule FA, Waltregny D, Castronovo V (2001) Increased expression of galectin-1 in carcinoma-associated stroma predicts poor outcome in prostate carcinoma patients. J Pathol 193:80-7

8. Clausse N, van den Brule F, Waltregny D, Garnier F, Castronovo V (1999) Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion. Angiogenesis 3:317-25

9. He J and Baum LG (2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. J Biol Chem (in press)

Figure Legends

Fig. 1. Protein phosphatase 2A (PP2A) inhibitors selectively block galectin-1 induced death of T cells. Human CEM T cells were incubated with the indicated inhibitors for one hr prior to addition of galectin-1. Cell death was detected by staining with annexin V and propidium iodide, and results are presented as percent cell death relative to cells treated with the respective vehicle controls. While okadaic acid and calyculin may have weaker but overlapping effects on PP1, cantharidic acid is PP2A specific, so that all three PP2A inhibitors blocked T cell death induced by galectin-1. In addition, no inhibitory effect was observed with the selective PP1 inhibitor phosphatidic acid; the paradoxical increase in galectin-1 death may be due to the ability of phosphatidic acid to augment PP2A activity while inhibiting PP1.

Fig. 2. Expression of the ST3Gal I sialyltransferase enzyme masks core 1 O-glycans and decreases susceptibility of LNCaP cells to galectin-1 induced cell death. LNCaP cells were transiently transfected with cDNA encoding the ST3Gal I enzyme, or with vector alone. After two weeks of growth in selective media, bulk cultures were analyzed by flow cytometry for reactivity with PNA, a lectin that recognizes the Galβ1,3GalNAc sequence on core 1 O-glycans. Sialylation of the Galβ1,3GalNAc sequence to yield the SAα2,3Galβ1,3GalNAc sequence masks the PNA binding site and demonstrates function of the ST3Gal I enzyme in the cells. The left panel demonstrates a significant decrease in PNA staining of the LNCaP cells transfected with the ST3Gal I cDNA (LNC-ST3), compared to parental LNCaP cells transfected with vector alone. The right panel shows the susceptibility to galectin-1 death of the same cultures that were phenotyped for PNA binding. The LNC-ST3 cells showed a 30% decrease in susceptibility to galectin-1, compared to the parental LNCaP cells transfected with vector alone.

Fig. 3. Galectin-3 competes with galectin-1 for binding to the cell surface. Human Jurkat T cells were incubated with biotinylated galectin-1 with or without unlabeled galectin-3, and the amount of galectin-1 bound to the cell surface quantified by addition of avidin-FITC and analysis by flow cytometry.

Baum, Linda G.

Fig. 1

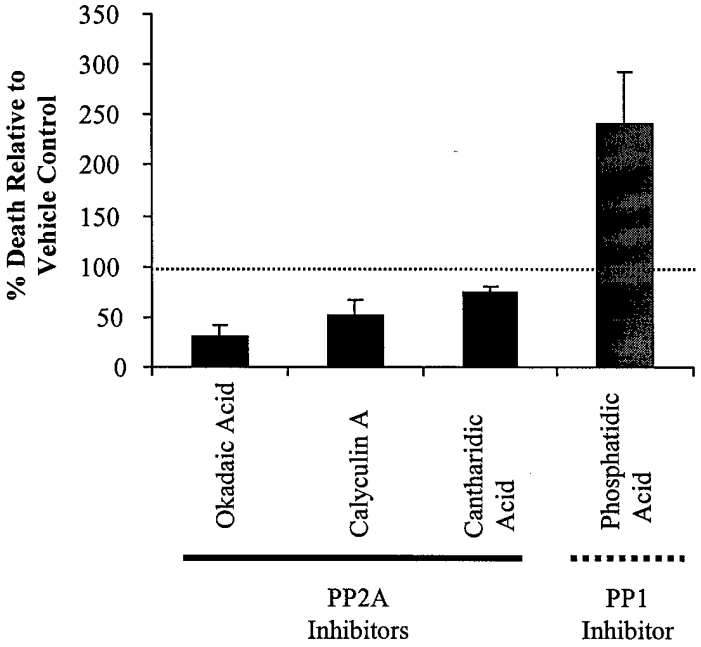
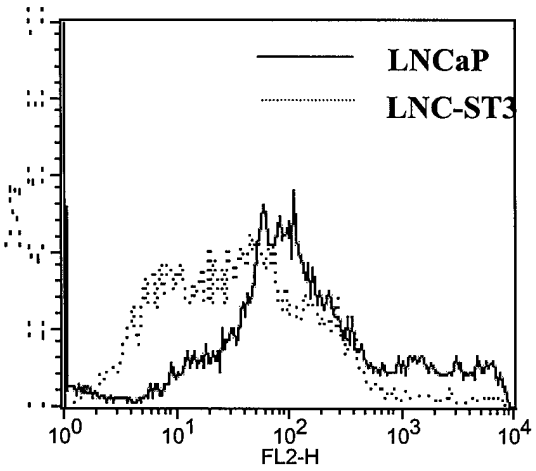


Fig. 2



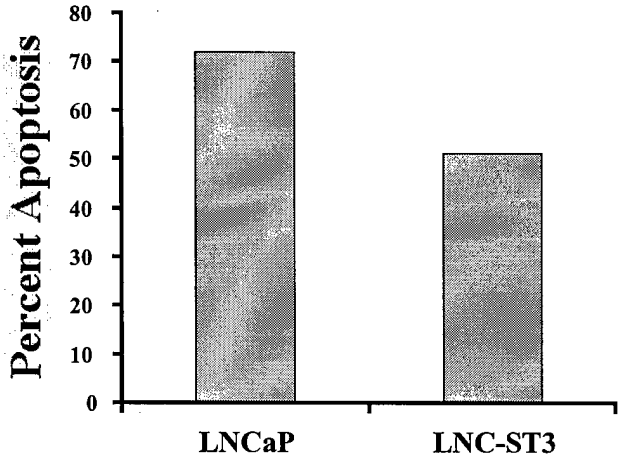
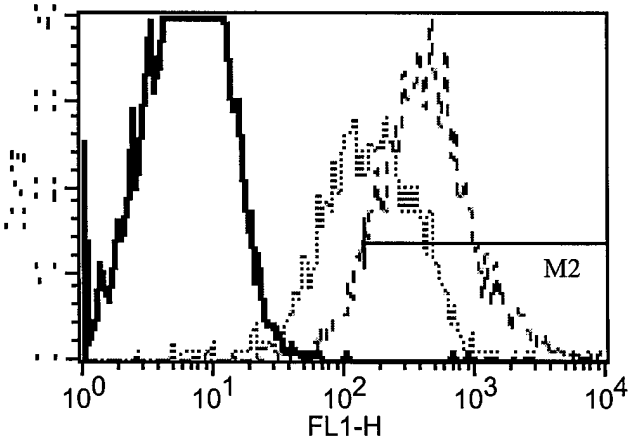


Fig. 3



Treatment and % in M2 gate

No gal-1	0%
10 uM gal-3/10 uM gal-1	52%
10 uM gal-1	92%

Table 1

Cell line	Galectin-1	Galectin-3
LNCaP	No	No
PC3	Yes	Yes
DU145	Yes	Yes
LAPC-4	No	Yes

Galectin-1 and galectin-3 expression were examined in human prostate cancer cell lines by Western blotting. The LAPC-4 cell line was obtained from Dr. Charles Sawyers, Dept. of Medicine, UCLA School of Medicine.

JBC Papers in Press. Published on November 14, 2003 as Manuscript M311183200

Presentation of galectin-1 by extracellular matrix triggers T cell death

Jiale He and Linda G. Baum [1,2]

Dept. of Pathology and [2] Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA 90095, USA

[1] To whom correspondance should be addressed

Phone 310-206-5985

Fax 310-206-0657

lbaum@mednet.ucla.edu

Running title: Cell-cell and cell-matrix contact in galectin-1 cell death

Keywords: cell death/ galectin-1/ extracellular matrix/ glycosylation

Abbreviations: CHO, Chinese Hamster Ovary; ECM, extracellular matrix; FITC, fluorescein isothiocyante; HRP, horseradish peroxidase; PBS, phosphate buffered saline; P.I., propidium iodide; Treg, regulatory T cells; UDP-Gal, UDP-galactose.

Summary

Apoptotic elimination of T cells at sites of inflammation or infiltration into tumors limits an effective immune response. T cell apoptosis can be initiated by a variety of triggers, including galectin-1, a soluble, secreted lectin that binds to oligosaccharide ligands on cell surface glycoproteins, or to oligosaccharide ligands on extracellular matrix glycoproteins in tissue stroma. Although galectin-1 has no transmembrane domain and is secreted from cells that make it, it is not clear if galectin-1 functions as a soluble death trigger *in vivo*. We examined the ability of stromal cells secreting galectin-1 to kill T cells. While the stromal cells synthesized abundant galectin-1, the majority of the galectin-1 remained bound to the cell surface, and stromal cell-associated galectin-1 killed bound T cells. In contrast, insufficient amounts of functional galectin-1 were released from the stromal cells into the media to kill T cells in the absence of contact with stromal cells. However, when stromal cells were grown on Matrigel, a mixture of extracellular matrix proteins, or on permeable membranes above Matrigel, secreted galectin-1 bound to Matrigel and killed T cells without stromal cell contact. Ten-fold less galectin-1 on Matrigel was sufficient to kill adherent T cells, compared to soluble galectin-1. These results demonstrate that galectin-1 in extracellular matrix is able to directly kill susceptible T cells. As increased galectin-1 deposition in tumor stroma occurs with tumor progression in various types of cancer, galectin-1 in stroma may act locally in the apoptotic elimination of infiltrating T cells during an immune response.

Introduction

Various factors regulate lymphocyte survival. This regulation may have positive effects, e.g. prevention of self-recognition and autoimmune disease, or negative effects, e.g. cancer cells can kill infiltrating lymphocytes that would attack the tumor (1-3). Galectins are a family of mammalian lectins with a variety of immunoregulatory functions, including control of lymphocyte death (4-9). Galectin-1, the first member of the family to be described, has a broad repertoire of immunoregulatory effects. Galectin-1 regulates the inflammatory responses of neutrophils, mast cells, and macrophages, and also associates with components of the complement system (10,11). Galectin-1 induces apoptosis of macrophages, thymocytes, T cells and B cells (9). Pircher et al detected an increase in galectin-1 synthesis after activation of murine T cells, and suggested that galectin-1 can act as an autocrine negative regulatory "cytokine", killing T cells to terminate an immune response (12). Galectin-1 is highly expressed in $CD4^+/CD25^+$ regulatory T (Treg) cells that suppress immune responses, compared to conventional CD4 cells (13). *In vivo*, galectin-1 therapy ameliorated disease in models of hepatitis, nephritis, arthritis, inflammatory bowel disease and multiple sclerosis (9,14).

Galectin-1 is expressed in a variety of cell types, including thymic epithelial cells, endothelial cells, dendritic cells, macrophages, fibroblasts and bone marrow stromal cells (4-6,8,9,15,16). There is increased galectin-1 expression in many types of cancer, including colon, breast, ovary and prostate carcinomas, and aggressive glioblastomas (8), and increased accumulation of galectin-1 in stroma surrounding tumor cells in ovarian and prostate carcinoma (17,18). Numerous ligands for galectin-1 have been described in different tissues; in extracellular matrix, galectin-1 binds to laminin, fibronectin and vitronectin, and is proposed to facilitate tumor cell invasion and migration through stroma (4,5,8,17-19). While galectin-1 may function

in tumorigenesis and metastasis, galectin-1 expression by tumors may also modulate the immune response to the tumor (4).

The mechanisms by which galectin-1 mediates these immunomodulatory effects have not been elucidated. In particular, it is not known whether galectin-1 can diffuse away from the cell that secretes it, to act as a soluble "cytokine", or whether galectin-1 requires direct cell-cell contact to exert its effects. In the former case, secretion of large amounts of soluble galectin-1 by tumors could have a global immunosuppressive effect. In the latter case, while galectin-1 synthesis may be increased in a tumor, the immunosuppressive effects would be limited to the vicinity of the tumor. Moreover, while several studies have documented accumulation of galectin-1 in extracellular matrix (ECM), the ability of ECM-associated galectin-1 to effect T cell death is not known.

Experimental Procedures

Chemicals and reagents

Recombinant human galectin-1 and polyclonal rabbit antiserum to human galectin-1 were prepared as described (15). The following reagents were purchased as indicated: annexin V/ propidium iodide (P.I.), galectin-3 (R&D Systems, Minneapolis, MN); dithiothreitol (Fisher Scientific, Fairlawn, NJ); 10X PBS, bovine serum albumin (Sigma Chemical, St Louis, MO); Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ); horseradish peroxidase (HRP) - chromogen kit (Biomedia, Foster, CA); Matrigel (BD Biosciences, Franklin Lakes, NJ); mouse anti-rabbit-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA); Texas Red-goat anti-rabbit IgG, FITC-annexin V (Molecular Probes, Eugene, OR); sulfo-NHS-biotin (Pierce, Rockford, IL).

Cells and culture conditions

The BW5147Pha^{R}2.1 cell line (Pha^{R}2.1), gift of Dr. M. Pierce, and the θ35 thymic stromal epithelial cell line, gift of Dr. K. Dorshkind, were cultured as described (20). Chinese Hamster Ovary (CHO) cells and Lec8-CHO (American Type Tissue Collection, Rockville, MD) were grown in α-MEM (GIBCO, Carlsbad, CA) with 10% fetal bovine serum. For transwell experiments, 4.5 x 10^5 θ35 cells, CHO, or Lec8 cells in 3 ml media were plated in the bottom chamber of 6-well plates (Costar, Corning, NY) for 24 h. To assess T cell death, viability and proliferation, fresh media was added to the stromal cells, and 2.25 × 10^5 Pha^{R}2.1 cells in 1.5 ml media were placed in the upper inserts (0.4 μm pore size, Falcon/Becton Dickinson); cell counts and trypan blue exclusion assays were performed at the indicated times. Pha^{R}2.1 cell death was assessed by flow cytometry using annexin V and P.I., as described (20).

To deposit galectin-1 on Matrigel, 2 x 10^5 stromal cells in 1.5 ml media were plated directly on solidified Matrigel, or placed in upper inserts, and 3 ml of medium was added to the lower wells over 100 µl solidified Matrigel on glass cover slips (Fisher, Pittsburgh, PA). 100 µl of liquid Matrigel was aliquoted onto coverslips in each well at 4°C. Plates were brought to room temperature to solidify the gel, and the gel air-dried for 1h. 100 µl of media was added to the solidified gel to keep the gel hydrated. Galectin-1 bound to Matrigel was detected by immunohistochemistry and quantitated by ELISA (below).

To bind recombinant galectin-1 or galectin-3 directly to Matrigel, 100 µl of galectin at the indicated concentration in PBS/0.1% BSA was added to the surface of the solidified Matrigel for one h. The Matrigel was washed once with PBS prior to T cell binding assays. No unbound galectin-1 or galectin-3 was detected in the wash buffer after Matrigel binding, indicating that all added galectin bound to the Matrigel.

Cell conjugate assays and confocal microscopy

To assess T cell death by cell-cell contact, 10^5 Pha^R2.1 cells were incubated for one h with subconfluent (~50%) monolayers (approximately 2 x 10^5 cells) of θ35 cells, CHO, and Lec8 cells plated on coverslips in 6-well plates. To assess T cell death by T cell-matrix contact, 10^5 Pha^R 2.1 cells were added to Matrigel solidified on coverslips and allowed to bind the Matrigel for 1 h. Unbound cells were removed by washing once with PBS. 100 µl of annexin V-FITC/P.I. in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) was added to the wells for 20 min, 20°C. Coverslips were washed with PBS, fixed with 2% paraformaldehyde, 30 min, 4°C, washed with PBS, quenched with 0.2 M glycine in PBS for 10 min, 4°C, and mounted onto slides with 25 µl Prolong Anti-fade mounting medium (Molecular Probes, Eugene OR).

For immunohistochemistry, cell conjugates or cell-Matrigel layers were prepared on coverslips as above, blocked with 10% goat serum, incubated with either anti-galectin-1 antiserum or with normal rabbit serum, all diluted 1:100 in PBS with 2% goat serum. After washing, bound antibody was detected with Texas Red-goat anti-rabbit IgG for 1h, 20°C for fluorescence microscopy, or with mouse anti-rabbit-HRP (1:200) and AEC for light microscopy. After washing, coverslips were mounted to slides as above.

For fluorescence microscopy, samples were excited at 488 and 568 nm with argon and krypton lasers, for FITC and Texas Red or P.I. respectively, and the light emitted between 525-540 nm was recorded for FITC and above 630 nm for Texas Red and P.I. Slides were visualized on a Fluoview laser scanning confocal microscope (Olympus America Inc, Melville, NY), using the 100X objective. Dual emission fluorescent images were collected in separate channels. Images were processed using the Fluoview image analysis software (version 2.1.39). To compensate the microscope, single stains with corresponding negative controls were performed. Quantification of $Pha^R2.1$ cell death was evaluated in 50-100 conjugates randomly selected in 5-8 microscopic fields for each experiment. The percent annexin V- positive T cells was calculated as the number of annexin V-positive T cells over the total number of adherent T cells.

ELISA assay for galectin-1

Anti-galectin-1 IgG was purified from rabbit polyclonal anti-galectin-1 antiserum on a Proteus Protein A spin column (Pro-Chem, Acton, MA). Purified anti-galectin-1 IgG was diluted in PBS to 10 μg/ml, and 100 μl/well added to 96-well enhanced protein-binding ELISA plates (Immulon 2, Franklin, MA) overnight, 4 °C. Wells were washed three times with wash buffer (1X PBS, 0.05% Tween 20) and blocked with blocking buffer (10% fetal bovine serum in PBS) 1 h, room temperature. After blocking, recombinant galectin-1, conditioned media, or cell lysates were

added for 1 h at room temperature. Wells were washed three times, biotinylated anti-galectin-1 IgG (10 μg/ml in blocking buffer) prepared as in (21) was added for 1 h, and wells were washed three times. Streptavidin-HRP (1:500 in blocking buffer) was added for 30 min, room temperature. After washing, 200 μl of 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD) was added to each well, and the absorbance of each well read at 490nm using a microplate reader (Bio-Rad Model 550, Hercules, CA). OD values were converted to protein concentration based on a galectin-1 standard curve. To quantify galectin-1 bound to Matrigel, biotinylated anti-galectin-1 IgG was added directly to the Matrigel and allowed to bind for 1 h. Color was developed as above, and 200 μl aliquots were transferred to 96-well ELISA plates.

Statistical analysis

Data were analyzed by student's t test, one-way ANOVA analysis. Significance was considered at $P<0.05$ and data are presented as mean ± SEM.

Results

Direct T cell binding to cells that express galectin-1, such as human and murine thymic stromal cells and activated endothelial cells, can trigger T cell death (15,20). However, it has been suggested that galectin-1 can act as a cytokine or soluble factor *in vivo* to trigger T cell death and inhibit T cell proliferation (12,22). To directly examine whether cells that synthesize galectin-1 can kill T cells in the absence of cell-cell contact, we examined the ability of the murine θ35 thymic stromal cells to kill murine Pha^R2.1 T cells (Fig. 1A). We observed that >50% of Pha^R2.1 T cells in contact with θ35 cells became annexin V-positive within one h of binding to θ35 cells. The annexin V-positive cells also demonstrated membrane blebbing, and clustering of annexin V on apoptotic blebs, as we have described previously (20). In addition to annexin V staining, cells were also labeled with propidium iodide to detect the later stage of cell death characterized by loss of membrane integrity. 45% of the annexin V-positive cells were also labeled with propidium iodide (data not shown), demonstrating that several hallmarks of cell death were present after the T cells bound to the thymic stromal cells.

Pha^R2.1 cell death appeared to require direct contact with θ35 cells, as T cells on the plastic tissue culture surface adjacent to, but not in contact with, the θ35 cells showed no cell death above background (Figs. 1A, 1B). This suggested that the θ35 cells did not secrete sufficient gal-1 to kill nearby T cells that were not in direct contact with the θ35 stromal cells. In addition, T cell death was galectin-1 dependent, as death was virtually abolished by the addition of anti-galectin-1 antiserum (Fig. 1A).

To examine the requirement for contact between galectin-1 expressing cells and T cells for T cell death, we plated θ35 thymic stromal cells in the bottom wells of transwell plates, and added Pha^R2.1 T cells to the top wells. This would allow any galectin-1 secreted by the θ35 cells

to diffuse through the semi-permeable membrane separating the upper and lower wells, but would not allow T cell - stromal cell contact. In contrast to experiments in which direct T cell – stromal cell contact triggered T cell death within one h (Fig. 1), we detected no loss of T cell viability when T cells were grown in the upper wells of transwell plates above θ35 cells for 24, 48, or 72 h; the viability of T cells cultured over θ35 cells was essentially identical to the viability of T cells cultured over bottom wells containing media alone (Table I). Similar results were seen at 1, 3, 6, 12 and 144 h of culture (data not shown).

We then examined CHO and Lec8 cells as galectin-1 secreting cells. CHO cells make abundant galectin-1 that is secreted from the cell and binds back to oligosaccharide ligands on the cell surface (23). Lec8 cells are a CHO mutant lacking UDP-galactose (UDP-Gal) transporter activity (24); as no galactose residues are added to cell surface glycoproteins or glycolipids, there are no available oligosaccharide ligands for galectin-1 on the surface of Lec8 cells, and all the galectin-1 is secreted into the media (23). θ35, CHO and Lec8 cells all make abundant galectin-1, between 40-60 μg/mg of total cellular protein; we detected galectin-1 on the surface of θ35 and CHO, but not Lec 8 cells (data not shown).

>70% of Pha^R2.1 T cells that adhered to CHO cells became annexin V positive within one h of contact with CHO cells. In contrast, we detected no annexin V binding to T cells in contact with Lec8 cells that had no detectable cell surface galectin-1 (Figs. 1C,D). As we had observed with the θ35 cells, in addition to annexin V binding, we observed membrane blebbing of T cells in contact with CHO cells (Fig. 1D), but no membrane blebbing of T cells in contact with Lec8 cells. When the T cells were added to the CHO cells, 61% of the annexin V-positive cells also labeled with propidium iodide, indicating loss of membrane integrity (data not shown).

Again, as we observed with θ35 cells, there was no loss of viability of Pha^R2.1 T cells in the upper wells of the transwell plates when either CHO or Lec8 cells were plated in the bottom wells, compared to bottom wells containing media alone (Table 1). We measured the amount of galectin-1 secreted from θ35, CHO and Lec8 cells into the media in the bottom of the transwell plates (Table 1). The θ35 cells secreted barely detectable amounts of galectin-1 into the media, while both the CHO and Lec8 cells secreted appreciable quantities of galectin-1 into the media (47 and 107 μg/ml, respectively). Indeed, galectin-1 secretion by Lec8 cells was increased compared to CHO cells, because the Lec8 cells did not bind back the secreted galectin-1. However, while these cells made abundant galectin-1, the amount of soluble galectin-1 secreted by either CHO or Lec8 cells into the media was not sufficient to reach the concentration required for Pha^R2.1 T cell death. We have determined that concentrations above 150 μg/ml (10 μM) soluble galectin-1 are optimal for triggering T cell death, most likely because the K_d of the galectin-1 dimer is in this range, and dimeric galectin-1 is required to induce death (15,19). Thus, the requirement for stromal cell – T cell contact demonstrated in Fig. 1 may reflect an ability of the stromal cells to concentrate the galectin-1 at the cell-cell interface, rather an active process by stromal cells in triggering T cell death.

To confirm that soluble galectin-1 could kill Pha^R2.1 cells in the transwell system, we added increasing concentrations of soluble galectin-1 to the lower wells of the transwell plates, and measured death of T cells in the upper wells. As shown in Fig. 2A, no death of Pha^R2.1 T cells in the upper wells of the transwell chambers was observed until the concentration of soluble galectin-1 in the wells was in excess of 150 μg/ml, which was also the concentration at which T cell agglutination began to occur, confirming multimeric binding of galectin-1. In these experiments, appreciable T cell death was seen at galectin-1 concentrations of 380 μg/ml and

above. Thus, very high concentrations of galectin-1 would have to be released from stromal cells in the bottom wells to kill T cells only 0.9 mm across the transwell plate.

In addition, there was no positive or negative effect on T cell proliferation over 72 h by galectin-1 secreted from stromal cells. The numbers of Pha^R2.1 cells in the upper wells of the transwell plates were essentially identical at 24, 48 and 72 h, whether the lower wells contained CHO cells, Lec8 cells or no stromal cells (Fig. 2B). Galectin-1 stimulates neural and hepatic cell proliferation at concentrations as low as 5 pg/ml (25,26); while CHO and Lec8 cells secreted 40-100 μg/ml of galectin-1 (Table 1), we detected no effect on T cell proliferation.

Thus, the ability of stromal cells to kill T cells via galectin-1 may relate to the ability of the stromal cell surface to concentrate and present galectin-1. As several extracellular matrix glycoproteins, such as laminin, fibronectin and vitronectin, bind galectin-1, cells secreting galectin-1 could also deposit the lectin on surrounding extracellular matrix; increased galectin-1 accumulation in tumor stroma could kill infiltrating T cells (17,18). To examine galectin-1 secretion into extracellular matrix, we used Matrigel, which contains laminin, collagen IV, entactin, nidogen, and heparan sulfate proteoglycans. By immunoblot analysis, we did not detect any endogenous galectin-1 in Matrigel (data not shown).

CHO and Lec8 cells were plated on Matrigel, and the deposition of galectin-1 on the Matrigel detected immunohistochemically. Abundant galectin-1 was deposited on Matrigel cultured with either CHO or Lec8 cells (Fig. 3A). We added T cells to plates containing Matrigel, and either CHO or Lec8 cells. Matrigel coated by galectin-1 produced by CHO cells killed a significant fraction of bound Pha^R2.1 T cells (Fig. 3B). Importantly, T cells analyzed in Fig. 3B were adjacent to, but did not appear to be in contact with, the adherent CHO or Lec8 cells (Fig. 3C). Thus, galectin-1 in extracellular matrix, as well as on the cell surface, could

trigger T cell death, although the level of T cell death on the matrix was less than that observed for direct T cell-CHO cell contact (Fig. 1A,C).

Surprisingly, we also observed T cell death when T cells were added to Matrigel coated by galectin-1 from Lec8 cells (Fig. 3B), although no death above background was observed when T cells bound directly to Lec8 cells (Fig. 1C). Growth of Lec8 cells directly on Matrigel apparently allowed the secreted galectin-1 to bind to glycoconjugate ligands in the extracellular matrix and retain carbohydrate binding activity (Fig. 3A). On Matrigel coated with galectin-1 from either CHO or Lec8 cells, galectin-1 was responsible for T cell death, since there was no death of T cells plated on Matrigel alone, and anti-galectin-1 antiserum reduced T cell death to background levels.

To exclude the possibility that death of T cells on galectin-1 coated Matrigel involved a brief T cell-stromal cell contact step prior to T cell adhesion to the matrix, we plated CHO or Lec8 cells in the upper wells of transwell plates with a layer of Matrigel in the bottom wells. Galectin-1 secreted by CHO or Lec8 cells diffused through the membrane in the upper chamber to deposit on the Matrigel (65 and 88 μg/ml, respectively, Fig. 4A). We examined the ability of galectin-1 coated Matrigel to kill adherent Pha^R2.1 T cells (Fig. 4A). Galectin-1 secreted by both CHO or Lec8 cells killed T cells bound to Matrigel. Of note, galectin-1 secreted from CHO or Lec8 cells killed the adherent T cells at surface concentrations of 2.6-3.5 $\mu g/cm^2$, or 65-88 μg/ml of Matrigel, a concentration of galectin-1 dramatically lower than the concentration of soluble galectin-1 required to kill the Pha^R2.1 T cells (Fig. 2A).

To determine the minimal amount of galectin-1 on Matrigel required to trigger T cell death, we coated solidified Matrigel with recombinant galectin-1. We observed T cell death when the amount of galectin-1 was as low as 1.7 $\mu g/cm^2$ (45 μg/ml) (Fig. 4B); in contrast, a

comparable level of T cell death required approximately 500 μg/ml of soluble galectin-1 (Fig. 2A). As a control, we examined the ability of galectin-3 to coat Matrigel and kill T cells; while galectin-3 bound to Matrigel, there was no death of T cells on galectin-3 coated Matrigel at concentrations as high as 100 μg/ml (Fig. 4B).

These results demonstrate that T cell contact with galectin-1 either on the surface of another cell, or on extracellular matrix in the absence of stromal cells, can kill adherent T cells. Moreover, the amount of galectin-1 secreted by CHO and Lec8 cells was sufficient to kill adherent T cells when the galectin-1 was presented on the surface of the extracellular matrix, while we observed no T cell death when galectin-1 was secreted from CHO or Lec8 cell into media surrounding the T cells.

Discussion

Galectin-1 participates in development, in immune system homeostasis, and in tumor progression (4-9). Pharmacologic administration of galectin-1 is effective in decreasing T cell responsiveness to antigens and inducing T cell apoptosis in several autoimmune disease models (9,14); however, in these models, immunosuppressive effects were not seen unless galectin-1 was administered at very high doses, typically 10 mg/kg, usually via an intraperitoneal route.

In contrast, while galectin-1 is very abundant in many cells and tissues (e.g. 35-40 μg/gm wet tissue in the spleen) (27), the serum concentration of galectin-1 in healthy women is only approximately 100 ng/ml (roughly equivalent to 420 μg total serum galectin-1 in a 60 kg person), while patients with ovarian carcinoma (a type of tumor that expresses galectin-1 at high levels compared to normal tissue) have even lower serum levels of galectin-1, approximately 20 ng/ml (28). The reduced serum concentration of galectin-1 in ovarian carcinoma patients with tumors that synthesize high levels of galectin-1 may result from the increased deposition of galectin-1 on glycoproteins such as CA125 on the tumor cell surface, as well as increased galectin-1 deposition in the ovarian carcinoma-associated stroma (29). The relatively low serum concentrations of galectin-1 in both healthy controls and cancer patients, in the ng/ml range, are far lower than the concentration of soluble, recombinant galectin-1 required for T cell death in *in vitro* assays (150 μg/ml). Thus, while galectin-1 has been called a "cytokine" (12), it is unlikely that increased galectin-1 synthesis in tumors would result in sufficiently elevated serum levels of galectin-1 to cause systemic immunosuppression.

However, as galectin-1 produced by tumors may primarily deposit on the tumor cells and surrounding stroma, galectin-1 may have profound local effects on the immune response to the tumor. Our data demonstrate that acellular matrix can bind galectin-1 secreted from stromal cells

and kill adherent T cells within 60 min. While apoptotic death of tumor-infiltrating T cells has been observed, this has been attributed to T cell encounters with death ligands directly on the tumor cell surface (2), rather than in the tumor-associated stroma. Although the level of T cell death on galectin-1 coated matrix (25-40%) was typically lower than the level of T cell death initiated by T cell-CHO cell contact (50-75%) (Figs. 3,4 vs. Fig. 1), matrix-associated galectin-1 was sufficient to kill bound T cells; the membrane fluidity in cellular presentation of galectin-1 may facilitate the clustering of T cell glycoprotein counterreceptors that participate in galectin-1 induced cell death (21), compared to a more static presentation of galectin-1 on Matrigel.

Thus, the present study indicates that, even prior to encounter with tumor cells, T cells that bind galectin-1 in tumor-associated extracellular matrix may be triggered to die. As proposed by van den Brule and Castronovo, increased deposition of galectin-1 in carcinoma stroma may act as an "immunologic shield" surrounding tumor cells (18). As galectin-1 is secreted by many types of tumors, including glioblastoma, breast, and prostate carcinoma (4,8), immunotherapy approaches in these cancers may be thwarted by the rapid death of T cells as they encounter galectin-1-loaded matrix surrounding the tumor. Moreover, galectin-1 induced phosphatidylserine exposure on leukocytes is sufficient for phagocytosis of the cells by macrophages (30), suggesting that stromal macrophages could rapidly eliminate infiltrating T cells that encounter galectin-1.

It is intriguing that joint tissue stroma from patients with rheumatoid arthritis had decreased galectin-1 deposition compared to normal joint tissues, in contrast to the increased galectin-1 deposition seen in tumor stroma (31). Thus, the T cell infiltration and immune-mediated damage in rheumatoid arthritis may relate to the decreased level of galectin-1 in joint stroma. Of interest, $CD4^+CD25^+$ Treg cells that express high levels of galectin-1 (13) typically

require cell-cell contact to exert immunoregulatory function (3), consistent with our observation that galectin-1 bound to the cell surface or to matrix is most effective at killing T cells.

The movement of galectin-1 from cells that produce it to the extracellular matrix is likely facilitated by the relatively low affinity (micromolar) of lectins for saccharide ligands, resulting in a rapid on-off rate on the surface of the cell (19,32). However, the bivalency of galectin-1 may increase the likelihood of the secreted lectin remaining tethered to local glycoproteins, retaining the galectin-1 in the vicinity of cells that make it. Moreover, as the dissociation constant of the galectin-1 homodimer is in the micromolar range (19), the tethering of galectin-1 to matrix glycoproteins may increase the likelihood that the lectin remains in dimeric form, the form required to trigger T cell death. Of note, galectin-1 secreted from Lec8 cells will misfold and lose carbohydrate binding activity with a $T_{1/2}$ of approximately 10 h, while galectin-1 bound to saccharide ligands retains binding activity for weeks (19,23). Galectin-1 secreted from Lec8 cells onto Matrigel over 24-72h retained binding activity, suggesting that the secreted lectin bound fairly rapidly to saccharide ligands in the Matrigel. In addition, a galectin-1 concentration as low as 45 μg/ml was sufficient to kill T cells when the galectin-1 was presented on the Matrigel surface, while a ten-fold higher concentration of soluble galectin-1 was required to kill an equivalent fraction of T cells.

Extracellular matrix glycoproteins may thus serve three roles in mediating galectin-1 triggered T cell death. First, the extracellular matrix provides a rich source of saccharide ligands on glycoproteins such as laminin, fibronectin and vitronectin, which can contribute to maintaining galectin-1's carbohydrate binding activity. Second, matrix glycoproteins can concentrate galectin-1 secreted by surrounding cells to increase the fraction of galectin-1 molecules that are homodimers. Third, the matrix presents galectin-1 to the T cell surface in a

two-dimensional array, rather than in a three-dimensional space as when T cells encounter soluble galectin-1; this type of presentation may facilitate the interaction of galectin-1 with the large T cell surface glycoproteins CD43 and CD45 that form a glycocalyx surrounding the T cell, to increase the rate and/or duration of galectin-1 binding to the cell (32). Understanding the mechanism by which galectin-1 interacts with extracellular matrix in tissues is critical for designing effective therapeutic strategies for galectin-1 in localized and systemic autoimmune disease, and in manipulating the immune response to tumors.

Acknowledgements

We thank Leland D. Powell and Karen Pace for advice and review, Sorge Kelm and Nancy Perillo for insights, and Mabel Pang for technical assistance. Supported by NIH grant GM63281, Dept. of Defense Grant 17-02-1-0022, and the Cancer Research Institute (to LGB).

References

1. Palmer, E. (2003) *Nat. Rev. Immunol.* **3,** 383-391

2. Rivoltini, L., Carrabba, M., Huber, V., Castelli, C., Novellino, L., Dalerba, P., Mortarini, R., Arancia, G., Anichini, A., Fais, S., Parmiani, G. (2002) *Immunol. Rev.* **188,** 97-113.

3. McHugh, R.S., Shevach, E.M. (2002) *J. Allergy Clin. Immunol.* **110,** 693-702.

4. Perillo, N.L., Marcus, M.E., Baum, L.G. (1998) *J. Mol. Med* **76,** 402-412.

5. Hughes, R.C. (2001) *Biochimie* **83,** 667-676.

6. Rabinovich, G.A., Baum, L.G., Tinari, N., Paganelli, R., Natoli, C., Liu, F.T., Iacobelli, S. (2002) *Trends Immunol.* **23,** 313-320

7. Liu, F.T., Patterson, R.J., Wang, J.L. (2002) *Biochim. Biophys. Acta* **1572,** 263-273.

8. Danguy, A., Camby, I., Kiss, R. (2002) *Biochim Biophys Acta* **1572,** 285-293.

9. Hernandez, J.D., Baum, L.G. (2002) *Glycobiology* **12,** 127R-136R

10. Almkvist, J., Dahlgren, C., Leffler, H., Karlsson, A. (2002) *J. Immunol.* **168,** 4034-4041.

11. Correa, S.G., Sotomayor, C.E., Aoki, M.P., Maldonado, C.A., Rabinovich, G.A. (2003) *Glycobiology* **13,** 119-128.

12. Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L., Pircher, H. (1998) *Eur. J. Immunol.* **28,** 2311-2319.

13. McHugh, R.S., Whitters, M.J., Piccirillo, C.A., Young, D.A., Shevach, E.M., Collins, M., Byrne, M.C. (2002) *Immunity* **16,** 311-323.

14. Santucci, L., Fiorucci, S., Rubinstein, N., Mencarelli, A., Palazzetti, B., Federici, B., Rabinovich, G.A., Morelli, A. (2003) *Gastroenterology* **124,** 1381-1394.

15. Perillo, N.L., Pace, K.E., Seilhamer, J.J., Baum, L.G. (1995) *Nature* **378,** 736-739.

16. Gauthier, L., Rossi, B., Roux, F., Termine, E., Schiff, C. (2002) *Proc. Natl. Acad. Sci. U S A.* **99,** 13014-13019.

17. van den Brule, F., Califice, S., Garnier, F., Fernandez, P.L., Berchuck, A., Castronovo, V. (2003) *Lab. Invest.* **83,** 377-386.

18. van den Brule, F.A., Waltregny, D., Castronovo, V. (2001) *J. Pathol.* **193,** 80-87.

19. Cho, M., Cummings, R.D. (1995) *J. Biol. Chem.* **270,** 5198-5206.

20. Nguyen, J.T., Evans, D.P., Galvan, M., Pace, K.E., Leitenberg, D., Bui, T.N., Baum, L.G. (2001) *J. Immunol.* **167,** 5697-5707.

21. Amano, M., Galvan, M., He, J., Baum, L.G. (2003) *J. Biol. Chem.* **278,** 7469-7475.

22. Rabinovich, G.A., Iglesias, M.M., Modesti, N.M., Castagna, L.F., Wolfenstein-Todel, C., Riera, C.M., Sotomayor, C.E. (1998) *J. Immunol. 160,* 4831-4840.

23. Cho, M., Cummings, R.D. (1995) *J. Biol. Chem.* **270,** 5207-5212.

24. Stanley, P., Sundaram, S., Sallustio, S. (1991) *Glycobiology.* **1,** 307-314.

25. Fukaya, K., Hasegawa, M., Mashitani, T., Kadoya, T., Horie, H., Hayashi, Y., Fujisawa, H., Tachibana, O., Kida, S., Yamashita, J. (2003) *J. Neuropathol. Exp. Neurol.* **62,** 162-172.

26. Maeda, N., Kawada, N., Seki, S., Arakawa, T., Ikeda, K., Iwao, H., Okuyama, H., Hirabayashi, J., Kasai, K., Yoshizato, K. (2003) *J. Biol. Chem.* **278,** 18938-18944.

27. Ahmed, H., Fink, N.E., Pohl, J., Vasta, G.R. (1996) *J. Biochem. (Tokyo).* **120,** 1007-1019.

28. Allen, H.J., Sharma, A., Ahmed, H., Piver, M.S., Gamarra, M. (1993) *Tumour Biol.* **14,** 360-368.

29. Seelenmeyer, C., Wegehingel, S., Lechner, J., Nickel, W. (2003) *J. Cell Sci.* **116,** 1305-1318.

30. Dias-Baruffi, M., Zhu, H., Cho, M., Karmakar, S., McEver, R.P., Cummings, R.D. (2003) *J. Biol. Chem.* Jul 9 [Epub ahead of print, PMID: 12853445].

31. Harjacek, M., Diaz-Cano, S., De Miguel, M., Wolfe, H., Maldonado, C.A., Rabinovich, G.A. (2001) *J. Rheumatol.* **28,** 1914-1922

32. Brewer, C.F., Miceli, M.C., Baum, L.G. (2002) *Curr. Opin. Struct. Biol.* **12,** 616-623.

Figure Legends

Figure 1. Galectin-1 on the stromal cell surface kills adherent T cells. A, B) Pha^R2.1 T cells were added to subconfluent monolayers of θ35 thymic stromal cells for 1 h. Cell death was assessed by annexin V-FITC binding. The % annexin V^+ T cells in contact with or adjacent to θ35 cells was determined for 50-100 T cells in 5-8 microscopic fields. Top bar in A indicates % annexin V^+ T cells in the absence of θ35 cells. Death was inhibited by addition of anti-galectin-1 antiserum (anti-Gal-1). Panel B is a phase image of an adherent θ35 cell and a T cell (upper left); annexin V-FITC staining of the T cell (lower left); overlay of the phase and fluorescent images (upper right); galectin-1 expression by a θ35 cell (red) and annexin V-FITC binding by an adherent T cell (green) (lower right). C, D) Pha^R2.1 T cell death was assessed as above, on CHO or Lec8 cells. Top bar in C indicates % annexin V^+ T cells in the absence of CHO or Lec8 cells. Panel D shows phase images of T cells bound to CHO and Lec8 cells; numerous annexin V^+ T cells were bound to CHO cells, while T cells bound to Lec8 cells did not become annexin V^+. In A and C, values are the mean of triplicate samples in a representative experiment.

Figure 2. T cell death and proliferation. A) To ensure that soluble galectin-1 can cross the transwell membrane to kill T cells, increasing concentrations of recombinant galectin-1 were added to bottom wells, with Pha^R2.1 T cells in upper wells. After 24 h, the galectin-1 concentration in the media in the upper and lower wells was determined; values for the upper and lower wells were essentially equivalent and the mean is shown. Agglutination was observed by light microscopy, and T cell death was assessed by flow cytometry using annexin V and P.I. B) Secreted galectin-1 had no effect on Pha^R2.1 T cell proliferation. Pha^R2.1 cells were cultured in

the upper well inserts, with CHO, Lec8, or no cells in the bottom wells. $Pha^R2.1$ cell number was determined at indicated times. Results in A and B are the mean of triplicate samples.

Figure 3. Stromal cells secrete galectin-1 that binds to Matrigel and kills T cells. A) CHO or Lec8 cells were plated on solidified Matrigel for 72 h and galectin-1 detected by immunohistochemistry (red); no galectin-1 was detected in the absence of stromal cells (control). B) $Pha^R2.1$ cells were added to Matrigel on which CHO or Lec8 cells had grown for 72 h. After one h, % annexin V^+ T cells was determined; only T cells bound to Matrigel but not in direct contact with CHO or Lec8 cells (shown in C) were counted. Death was inhibited by anti-gal-1. Values are the mean of triplicate determinations. C) Annexin V binding to T cells on Matrigel adjacent to CHO or Lec8 cells. In each set, the top panel is a phase micrograph of a cluster of CHO or Lec8 cells (left) with individual T cells (right); the bottom panel is an overlay of phase and fluorescent images to delineate the edge of the CHO or Lec8 cluster and demonstrates FITC-annexin V binding to T cells.

Figure 4. Galectin-1 on Matrigel is sufficient to kill T cells in the absence of stromal cells. A) CHO, Lec8 or no stromal cells were placed in the upper well inserts over solidified Matrigel in the bottom wells for 48 h. The galectin-1 concentration bound to the Matrigel from CHO or Lec8 cells was determined (under respective bars). In parallel wells, 10^5 $Pha^R2.1$ cells were bound in the presence or absence of anti-gal-1 for one h, and % annexin V^+ T cells calculated. B) Indicated concentrations of galectin-1 or galectin-3 were bound directly to solidified Matrigel for one h. 10^5 $Pha^R2.1$ T cells were added to the Matrigel for one h, and % annexin V^+ T cells determined. In A and B, values are the mean of triplicate samples.

Figure 1

He and Baum

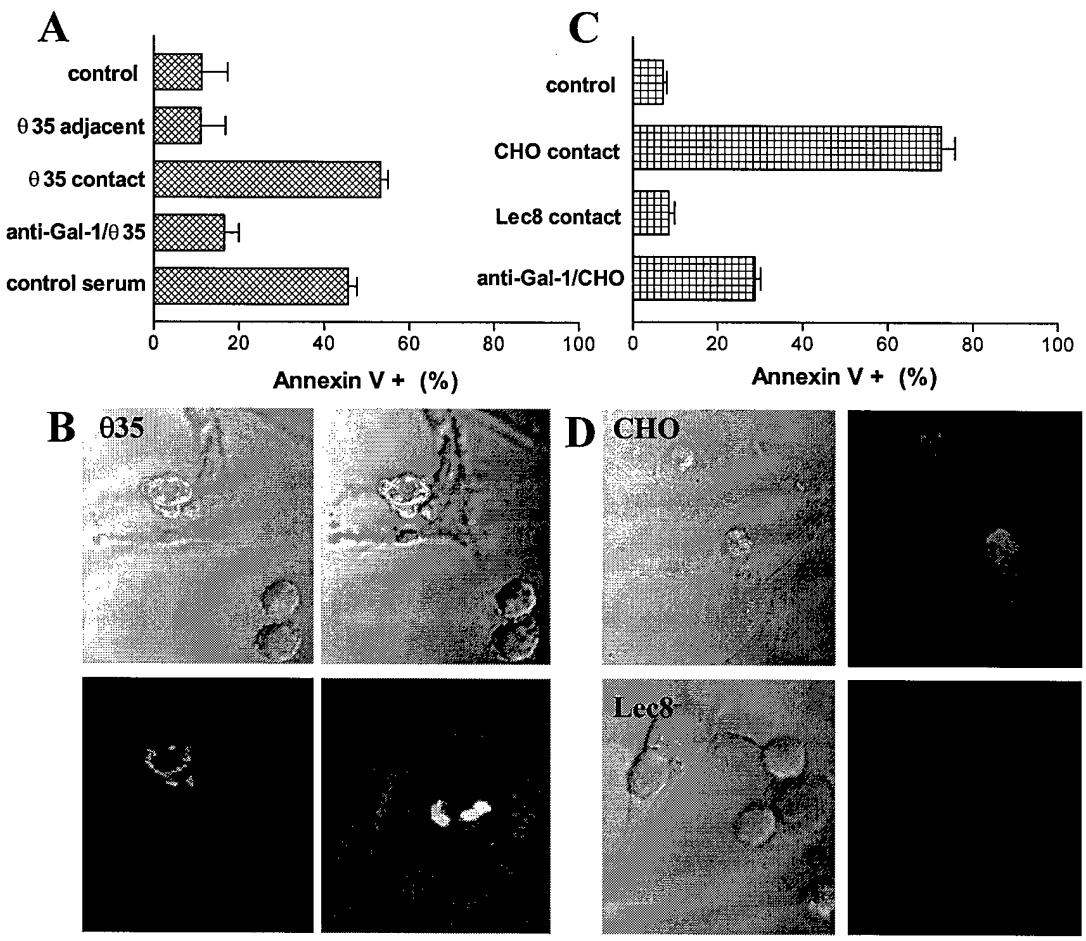


Figure 2 He and Baum

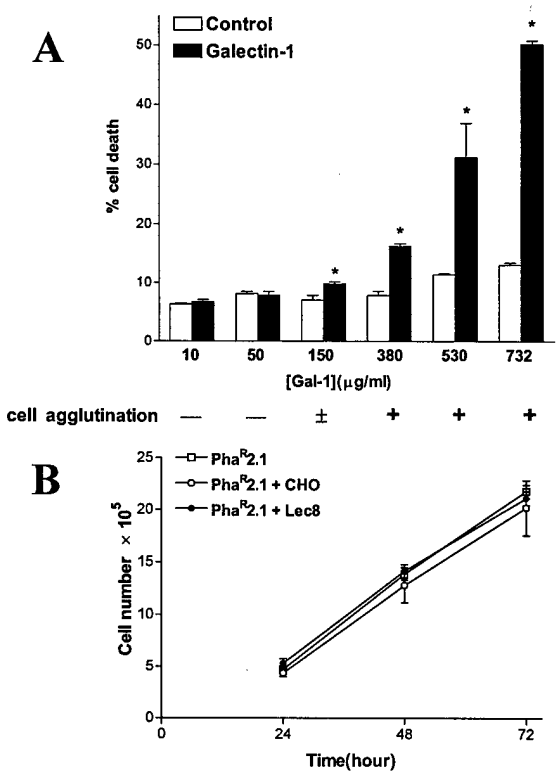


Figure 3 He and Baum

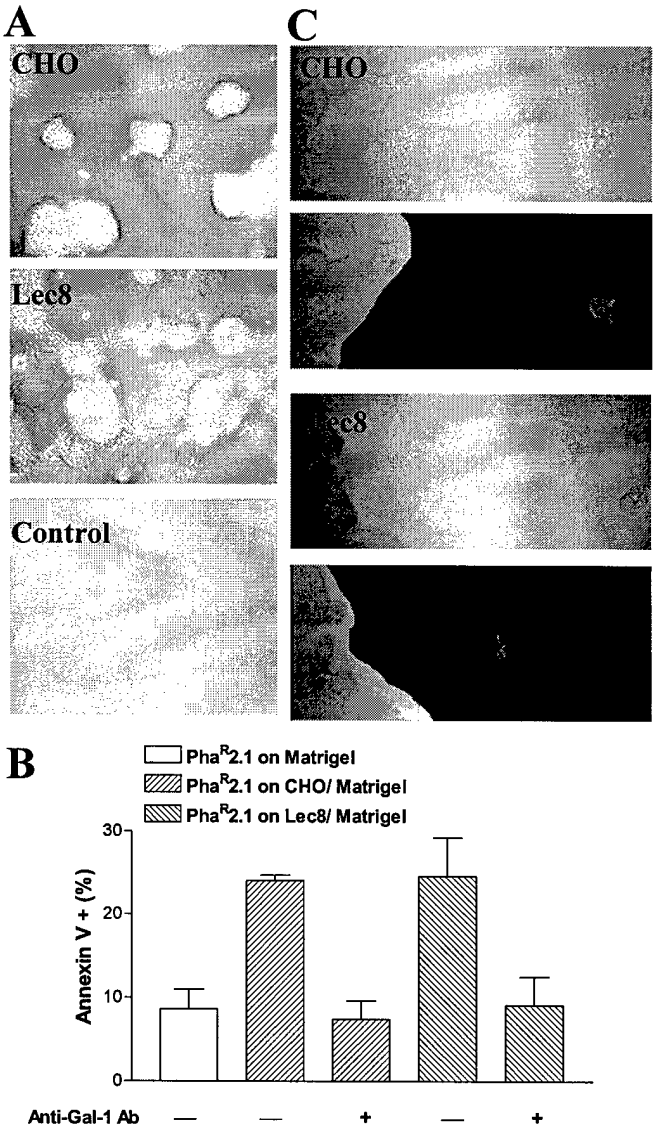


Figure 4

He and Baum

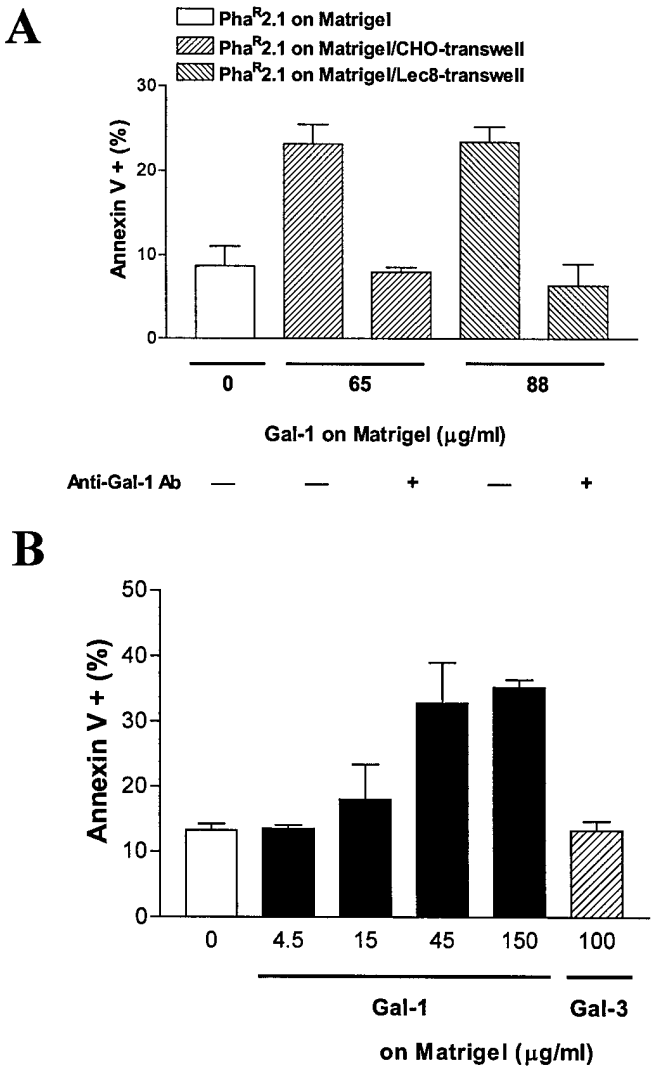


Table 1. Galectin-1 secretion and effect on T cell viability

	24h		48h		72h	
	Viab*	Gal-1^{+}	Viab	Gal-1	Viab	Gal-1
Media	100	—	100	—	100	—
θ 35	97.9	7.3	98.5	1.9	97.0	0.9
CHO	100.5	48.8	100.7	53.6	102.7	47.4
Lec8	100.6	40.5	99.1	79.5	99.7	106.8

* % Pha^{R}2.1 viability compared to control

\+ Galectin-1 concentration (μg/ml) in medium