Carbohydrate Research, 213 (1991) 345–352
Elsevier Science Publishers B.V., Amsterdam

Note

Purification in large amounts of β-D-galactoside-binding lectins from a murine thymic epithelial cell line*,†

Esther Bachar-Lustig, Yehudit Gan, and Yair Reisner‡
Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100 (Israel)

(Received February 26th, 1990; accepted for publication, in revised form, May 29th, 1990)

A family of animal lectins that bind β-D-galactosides has been isolated during the past decade from different tissues including heart[1], lung[2], muscle[3], liver[3], and fibroblasts[4] of diverse species (for review, see Sharon and Liss[5]). The β-D-galactoside-specific lectins can be solubilized without the aid of detergents, and are of similar molecular size. The most abundant forms of these lectins are dimers made up of 10–16 kD subunits, whereas other lectins are monomeric, and have a molecular mass of 17–20 or 34 kD[6]. Although these lectins exhibit considerable sequence homologies, they differ markedly in their preference for different D-galactose-containing disaccharides, suggesting a difference in their fine carbohydrate specificity[5].

Despite their wide distribution in animal tissues of different species, a specific cellular function cannot unequivocally be assigned to any of these lectins. A large body of evidence suggests that they are involved in a variety of cellular interactions in which the specific carbohydrate-binding site of the lectin binds a complementary sugar structure as a prelude to one of a number of biological activities (for review, see Barondes[7]). Considering that many plant lectins have been shown to stimulate mitosis of T lymphocytes, it is of interest to investigate the possible role of animal lectins in T cell differentiation.

The thymus is a critical site for T cell selection, differentiation, and self-recognition[8]. Lymphocyte precursors migrate from the bone marrow to the thymus, where they are processed in the presence of the thymic epithelial environment and subsequently migrate to peripheral lymphoid organs. The *in vivo* importance of these intrathymic interactions was shown by the lack of immunocompetence of athymic mice and neonatally thymectomized mice, as well as the association of human thymic agenesis with T cell immunodeficiency. Analytical studies of highly purified thymic stromal constituents at clonal and cellular levels indicated that epithelial cells provide fundamental signals for T cell maturation in the thymus, where lymphocytes learn to

* Dedicated to Professors Nathan Sharon and Toshiaki Osawa.

† Supported in part by a grant from the Israel Cancer Research Fund (New York).

‡ To whom correspondence should be addressed.

0008-6215/91/$03.50

discriminate between self and nonself[9–12]. However, the mechanisms by which these processes occur are mostly unknown.

In 1979, Barak-Briles *et al.*[13] found a lactose-binding lectin in calf thymus extracts. Subsequently, Levi and Teichberg[14] demonstrated the existence of a lactose-binding lectin in mouse thymic extract, preferentially located in a cell fraction enriched for epithelial cells. They also purified a similar lectin from a whole chicken thymus, which was found to have a M_r of 15300 Da when analyzed by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE), and an M_r of 30000 Da when analyzed by gel filtration[15].

However, as the mouse model is the most advanced and well-documented animal model for the study of *in vivo* and *in vitro* T cell differentiation, the murine thymic lectin can best help to elucidate whether these thymic lectins possess an immunological function. Purification of the murine thymic lectin in amounts sufficient to allow study of its biological function met with difficulties in the past, due to an inability to obtain large numbers of pure thymic epithelial cells in culture. In the present study, we overcame this problem by use of a thymic epithelial cell line (TEC) as a source for the lectin, which originates from SV-40 transformation of thymic cell monolayers and which, according to morphologic and biochemical criteria, appears to be of epithelial origin[16]. TEC soluble extracts were prepared as previously described by Raz and Lotan[17]. The extracts exhibited hemagglutinating lectin activity (assayed by agglutination of trypsin-treated rabbit erythrocytes) which was specifically inhibited by lactose (Fig. 1). The hemagglutinating activity was retained on a lactosyl-Sepharose column, and specifically eluted with 0.5M lactose (Fig. 2).

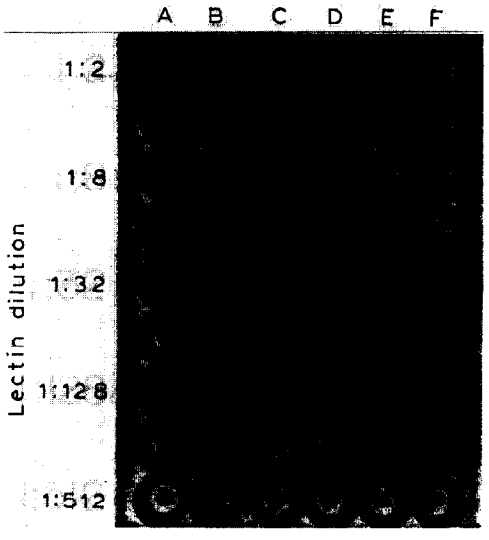

Fig. 1. Hemagglutination inhibition of TEC lectin. The following were added to a suspension of 2% erythrocyte in PBS (50 μL): (A) PBS, (B) cell extract alone, (C) cell extract with methyl α-D-mannopyranoside, (D) cell extract with D-galactose, (E) cell extract with lactose, and (F) cell extract with *N*-acetyl-D-galactosamine.

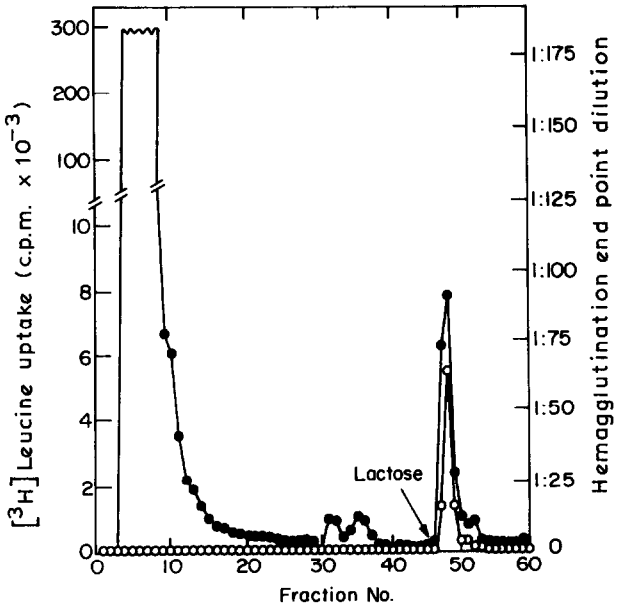

Fig. 2. Purification by affinity chromatography of thymic epithelial lectin from TEC cells prelabeled with [^{3}H]leucine: (-●-●-) [^{3}H]leucine (c.p.m.); (-o-o-) hemagglutinating activity.

The sugar specificity of the purified lectin was examined by inhibiting its hemagglutinating activity with various low-mol.wt. sugars. As shown in Table I, *N*-acetylactosamine and lactose were the most potent inhibitors of the purified lectin. D-Galactose, D-galactosamine, and methyl α-D-galactopyranoside, although less effective than lac-

TABLE I

Effect of sugars on hemagglutinating activity of the TEC lectin

Sugar	*Concentration for 50% inhibition (mM)*	*Minimal concentration[a] (mM)*
N-Acetyllactosamine	4.7	0.29
Lactose	18.75	2.34
D-Galactose	50	25
D-Galactosamine	50	12.5
N-Acetyl-D-galactosamine	<100	<100
Methyl α-D-galactopyranoside	50	25
D-Glucose	>100	>100
D-Fucose	>100	>100
L-Fucose	>100	>100
D-Mannose	>100	>100
Methyl α-D-mannopyranoside	>100	>100
Melibiose	>100	>100

[a] Minimal sugar concentration at which inhibition can be detected.

tose, also exhibited significant inhibitory activity. D-Glucose, D- and L-fucose, *N*-acetyl-D-glucosamine, D-mannose, methyl α-D-mannopyranoside, and D-galactosamine did not exhibit inhibitory activity at a 100 μM concentration.

The material eluted from the affinity column with lactose was determined by SDS–PAGE to consist of two polypeptides, having molecular masses of 14.5 and 35 kD, respectively (Fig. 3). Gel filtration by h.p.l.c. on TSK-SW 2000 revealed a major peak with an apparent M_r of ~30 kD. SDS–PAGE of this 30-kD fraction revealed a 14.5- and a 35-kD band (Fig. 4), suggesting that the TEC lectin preparation probably contains two different proteins, a monomer of 35 kD and a dimer made up of two 14.5 kD subunits. Further studies are required to separate these two proteins, but it seems from the mol. wts., the carbohydrate-binding specificity, and the antigenic cross-reactivity of the TEC lectin with other soluble lactose-specific lectins (see below) that they may be closely related.

Immunoblotting with polyclonal rabbit antiserum raised against the TEC lectin stained both the 14.5- and the 35-kD polypeptide bands (Fig. 5). A polyclonal rabbit antiserum, raised against a calf lung lactose-binding lectin, was cross-reactive with the two polypeptide bands. Similar cross-reactivity, as well as bands with the same molecular masses of 14.5 and 35 kD, was identified in extracts of normal murine thymic tissue (Fig. 6). The presence of 14.5- and 35-kD bands in normal thymic extracts that are stained by immunoblotting with the immune antisera specific for the TEC lectin strongly suggested that the TEC lectin, purified from an SV-40 transformed thymic epithelial cell line, does represent the lectin found in the relevant normal tissue. Such a hypothesis can only be definitively verified by comparing the amino acid sequences of both lectins, a difficult challenge owing to the scarcity of normal thymic epithelial cells.

Attempts to elucidate the function of such lectin(s) in T cell differentiation have in the past been largely unsuccessful, owing to the scarcity of thymic epithelial cells from which the lectin could be purified. In the present study, such lectin activity was found to be present in an SV-40 transformed murine thymic epithelial cell line, and was purified in large amounts by affinity chromatography on a lactosyl-Sepharose column. The physiological role of the TEC lectin in thymic differentiation can now be investigated in thymic cultures *in vitro*, both by direct application of the lectin and by use of the antibody specific for the lectin.

EXPERIMENTAL

Hemagglutination assay. — Trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes were prepared according to Nowak *et al.*[3]. The erythrocytes were stored at 4° as a 10% suspension in phosphate-buffered saline solution (PBS). Prior to the assay, they were diluted with 0.1M glycine in PBS to obtain a 2% suspension. Hemagglutinating activity was determined by diluting cell extracts in twofold steps in microtiter plates with a v-shaped bottom (Greiner). Each well contained cell extract (50 μL), diluted in PBS supplemented with 0.1% bovine serum albumin (BSA) (PBS–BSA), a 2% erythrocyte suspension (50 μL), and PBS–BSA (50 μL). The lowest extract dilution causing distinct hemagglutination was determined after incubation for 1 h at 25°.

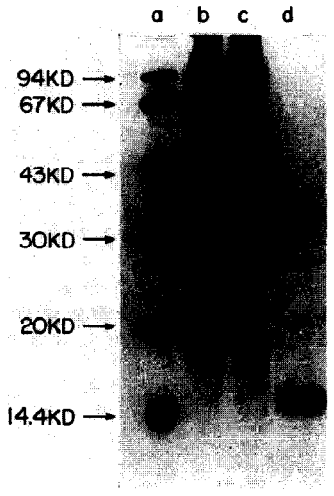

Fig. 3. SDS–PAGE of different fractions after purification of TEC lectin, stained with Coomassie Brilliant Blue: (a) molecular masses markers, (b) cell extract applied to the column, (c) effluent, and (d) elution with 0.5M lactose.

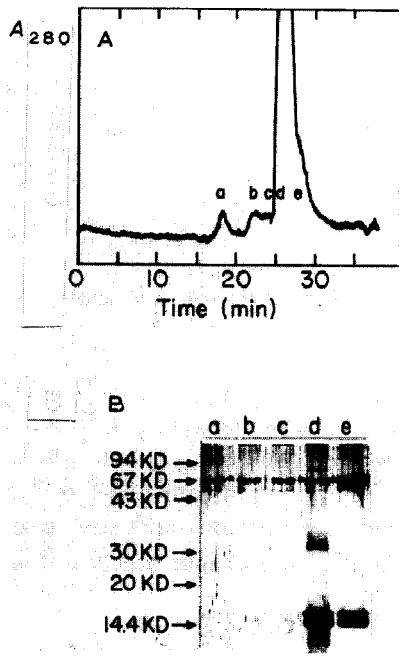

Fig. 4. (A) Gel filtration by h.p.l.c. of purified TEC lectin. (B) H.p.l.c. fractions were subjected to SDS–PAGE and stained with silver nitrate: a, b, c, d, and e are the peaks labeled in (A).

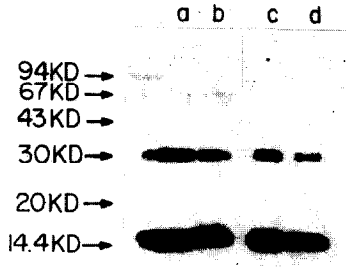

Fig. 5. Immunoblotting of purified TEC lectin. Two different affinity-purified TEC lectin preparations were subjected to immunoblotting with anticalf lung lectin rabbit antiserum (a,b) and anti-TEC lectin rabbit antiserum (c,d).

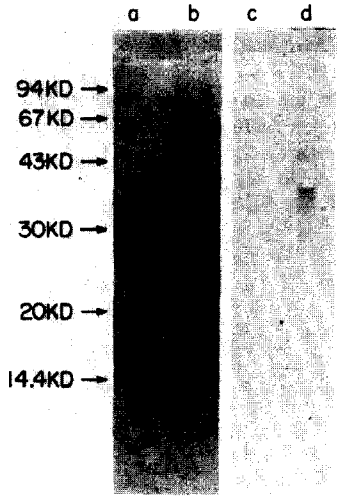

Fig. 6. Immunoblotting of a purified TEC lectin (a,c) and a murine thymic extract (b,d) with immune (a,b) and nonimmune (c,d) anticalf lung lectin antiserum.

When the effect of various sugars on hemagglutination was investigated, the sugars were diluted from a 0.3M stock solution by serial twofold dilutions in PBS–BSA (50 μL) in the microtiter plates. To each sugar-containing well cell, extract (50 μL) diluted in PBS–BSA was added in order to obtain a final dilution equivalent to four times the lowest concentration of the lectin causing hemagglutination in the absence of the tested sugar. After 1 h, the titer was recorded, and the lowest sugar concentration completely inhibiting hemagglutination determined.

Extraction and purification of the TEC lectin. — TEC cells were grown to confluence on 15-cm diameter dishes, collected, and washed in PBS. The cell pellet [1 g, (w/w)/mL] was suspended in extraction buffer consisting of 75mM Na_2HPO_4, 75mM NaCl, 4mM EDTA, 300mM lactose, and 14mM β-mercaptoethanol (pH 7.2). The cell suspensions were treated three times by sonication for 30 s each time, at 4°. The treated suspensions were centrifuged at 12 000*g* for 30 min at 4°, and the supernatants further centrifuged at 100 000*g* for 1 h. The resulting supernatants were extensively dialyzed

against PBS supplemented with 14mM β-mercaptoethanol, and then applied to an affinity column of lactosyl-Sepharose. After being loaded, the column was washed with PBS containing 14mM β-mercaptoethanol until no protein was detected in the effluent. The TEC lectin was eluted from the column by application of 0.5M lactose in PBS supplemented with 14mM β-mercaptoethanol. The elution of the TEC lectin was monitored by protein determination according to Bradford[18]. The fractions corresponding to the protein peak were pooled and extensively dialyzed against PBS containing 14mM β-mercaptoethanol.

Extract preparation from a normal thymus cell fraction enriched for epithelial cells. — Thymuses were excised, cut into small pieces in Dulbecco's modified Eagle's medium, and then gently pressed with sterile forceps to remove most of the lymphocytes. The latter operation was repeated until only very few lymphocytes remained in the medium. The lymphocyte-depleted thymuses were treated by sonication in extraction buffer (75mM Na_2HPO_4, 75mM NaCl, 4mM EDTA, 300mM lactose, and 14mM β-mercaptoethanol, pH 7.2; 1 g/mL) four times for 30 s at 4°. The treated suspension was centrifuged at 12 000*g* for 30 min at 4°, and the supernatant was further centrifuged at 100 000*g* for 1 h. The resulting supernatant was dialyzed extensively against PBS supplemented with 14mM β-mercaptoethanol.

Preparation of lactosyl-Sepharose resin. — The procedure of Porath and Ersson[19], modified by Teichberg *et al.*[20], was used. Briefly, sedimented Sepharose 4B beads (100 mL) were suspended in 0.5M Na_2CO_3 and washed several times until a pH of 11.0 was obtained. The gel was then filtered through a Büchner funnel. The drained gel was collected and resuspended in 0.5M Na_2CO_3 (100 mL), and divinyl sulfone (10 mL) was slowly added. The mixture was stirred gently for 70 min at 23°, filtered through a Büchner funnel, and washed several times with 0.5M Na_2CO_3. The drained gel was resuspended in a solution consisting of 10% lactose in 0.5M Na_2CO_3 (100 mL). The mixture was incubated for 15 h at 23° with gentle stirring, then filtered, and washed successively with 0.5M Na_2CO_3, water, and 0.05M phosphate buffer (pH 7).

Immunoblotting. — Immunoblotting was performed according to Gershoni *et al.*[21] The lectin extracts separated by SDS–PAGE, were blotted onto nitrocellulose filters in a gradient electric field (5–40 V). The filters were quenched for 2–16 h at 25° in PBS containing 2% BSA, and incubated for 2 h at 25° with rabbit antiserum directed against the TEC lectin (diluted 1:3000) or with a rabbit anticalf lung lectin antiserum diluted 1:5000. The filters were then washed three times with PBS containing 0.5% BSA and 0.02% Twin-20, and incubated with ^{125}I-goat antirabbit IgG for 1 h at 25°. After three washes, the filters were autoradiographed overnight, with Agfa X-ray films.

ACKNOWLEDGMENTS

The authors thank Dr. S. H. Barondes for a kind gift of anticalf lung lectin antiserum and Barbara Morgenstern for editorial assistance.

REFERENCES

1 A. de Waard, S. Hickman, and S. Kornfeld, *J. Biol. Chem.*, 251 (1976) 7581–7587.
2 J. T. Powell, *Biochem. J.*, 187 (1980) 123–129.
3 T. P. Nowak, D. Kobiler, L. E. Roel, and S. H. Barondes, *J. Biol. Chem.*, 252 (1977) 6026–6030.
4 C. F. Roff and J. L. Wang, *J. Biol. Chem.*, 258 (1983) 10 657–10 663.
5 N. Sharon and H. Liss, *Science*, 246 (1989) 227–234.
6 H. Leffler and S. H. Barondes, *Int. Symp. Lectins*, Nof Ginosar, Israel, (1989) 43.
7 S. H. Barondes, *Annu. Rev. Biochem.*, 50 (1981) 207–231.
8 O. Stutman, *Immunol. Rev.*, 42 (1978) 138–184.
9 R. M. Zinkernagel, *Immunol. Rev.*, 42 (1978) 224–270.
10 H. Wekerel, U. P. Ketelsen, and M. Ernst, *J. Exp. Med.*, 151 (1980) 925–944.
11 A. L. Goldstein, T. L. K. Low, G. B. Thurman, M. M. Zatz, N. Hall, J. Chen, S.-K. Hu, P. B. Naylor, and J. E. McClure, *Recent Progr. Horm. Res.*, 37 (1981) 369–415.
12 W. Born and H. Wekerel, *Eur. J. Immunol.*, 12 (1982) 51–59.
13 E. Barak-Briles, W. Gregory, P. Fletcher, and S. Kornfeld, *Cell. Biol.*, 81 (1979) 525–537.
14 G. Levi and V. I. Teichberg, *Immunol. Lett.*, 7 (1983) 35–39.
15 G. Levi and V. I. Teichberg, *Biochem. J.*, 226 (1985) 379–384.
16 L. H. Glimcher, W. E. Kruisbeek, and I. Green, *Scand. J. Immunol.*, 17 (1983) 1–11.
17 A. Raz and A. Lotan, *Cancer Res.*, 41 (1981) 3642–3647.
18 M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
19 J. Porath and B. Ersson, *Proc. Symp. New Approaches Inducing Nat. Immun. Pyrogenic Org.*, (1973) 101–108.
20 V. I. Teichberg, I. Silman, D. D. Beitsch, and G. Resheff, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 1383–1387.
21 J. M. Gershoni, F. E. Davis, and G. E. Palade, *Anal. Biochem.*, 143 (1984) 32–40.