Acta Medica Okayama

Volume 47, *Issue* 1 1993 *Article* 3

FEBRUARY 1993

Distribution of Lectin Receptors in the Human Hyperplastic Tonsil: Histochemical and Flow Cytometric Analyses

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Abstract

The distribution of lectin receptors in the human tonsil was studied using 16 biotinylated lectins. The avidin-biotin-peroxidase complex (ABC) method was used on frozen and paraffin-embedded tissue sections. Cell suspensions were also analysed by dual flow cytometry using respective fluorescein isothiocyanate-conjugated lectins and phycoerythrin-labeled anti-CD3 and anti-human immunoglobulin. Frozen sections fixed with acetone and paraffin-embedded materials fixed in three solutions were compared for lectin affinity; ethanol-fixed sections gave best results followed by frozen and buffered formalin-fixed ones, then nonbuffered formalin. Con-A, RCA-1, LcH, WGA, MPA, PHA, PSA, PNA, SJA and GSA-1 reacted with all tissue components of the tonsil in immunohistochemical studies, but binding intensity was fixative dependent. Binding of Lotus and BPA to lymphocytes was limited to germinal center lymphocytes. Other tissue components were also reactive but staining intensity was weaker in Lotus compared with BPA. SBA and DBA did not react with lymphocytes, but reacted with macrophages/histiocytes, vascular endothelia, and epithelial cells. LBA and LPA were constantly negative with all tissue components irrespective of fixatives. Flow cytometric analyses showed that all but three (DBA, LBA and LPA) partially or totally stained lymphocyte surfaces. Lotus receptors were expressed exclusively on B-lymphocytes.

KEYWORDS: lectins, ?histochemistry, flow cytometry, human tonsil

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*PMID: 8460551 [PubMed - indexed for MEDLINE]

Acta Med Okayama 47 (1) 13-19 (1993)

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The distribution of lectin receptors in the human tonsil was studied using 16 biotinylated lectins. The avidin-biotin-peroxidase complex (ABC) method was used on frozen and paraffin-embedded tissue sections. Cell suspensions were also analysed by dual flow cytometry using respective fluorescein isothiocyanate-conjugated lectins and phycoerythrin-labeled anti-CD3 and anti-human immunoglobulin. Frozen sections fixed with acetone and paraffin-embedded materials fixed in three solutions were compared for lectin affinity; ethanol-fixed sections gave best results followed by frozen and buffered formalin-fixed ones, then nonbuffered formalin. Con-A, RCA-1, LcH, WGA, MPA, PHA, PSA, PNA, SJA and GSA-1 reacted with all tissue components of the tonsil in immunohistochemical studies, but binding intensity was fixative dependent. Binding of Lotus and BPA to lymphocytes was limited to germinal center lymphocytes. Other tissue components were also reactive but staining intensity was weaker in Lotus compared with BPA. SBA and DBA did not react with lymphocytes, but reacted with macrophages/histiocytes, vascular endothelia, and epithelial cells. LBA and LPA were constantly negative with all tissue components irrespective of fixatives. Flow cytometric analyses showed that all but three (DBA, LBA and LPA) partially or totally stained lymphocyte surfaces. Lotus receptors were expressed exclusively on B-lymphocytes.

Key words : **lectins, histochemistry, flow cytometry, human tonsil**

Lectins are proteins or glycoproteins found in plants, animals or microorganisms which have specific binding affinity for carbohydrates (1). Several lectins have been extensively used to identify specialized cells in human and animal lymphoreticular tissues. However, the normal distribution of lectin receptors in human lymphoreticular tissues remains unclear. Discrepancies among reports could have been largely based on the difference of tissue processing and procedures (2-6). For example, Rose *et al.* (2), and Rose and Malchiodi (3) reported the selective binding of PNA to germinal center lymphocytes in frozen sections, and Wirbel *et al.* (6) observed that some paracortical lymphocytes were reactive. Hsu and Ree (4) found no reaction with lymphoid cells in paraffin sections.

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There has been little attempt to standardize fixation for the study with human lymphoreticular tissues. Most of the works have been performed on formalin fixed, paraffin-embedded tissues. Only a few have employed a range of different fixatives on both paraffin and frozen sections. Notably, Wirbel *et al.* (6) preferred formalin fixatives to acetone, chloroform, methanol or ethanol solutions because of the better localization of reaction product. They also have suggested that ethanol fixation totally destroys lectin affinity. But, Allison's findings supported the advantage of ethanol fixation over other fixatives (7). Leathem and Atkins reported that lectin binding was more easily demonstrable on frozen sections than paraffin sections because of attenuation of binding sites (8).

The present study was designed to resolve the discrep-

ancies found in the aforementioned studies regarding the influence of fixatives on lectin affinity and the controversy about lectin receptor distribution in human lymphoreticular tissues. We histochemically examined the binding of 16 selected lectins to human hyperplastic tonsils, frozen and acetone-fixed or fixed with buffered or nonbuffered formalin or ethanol. And also flow cytometric analysis was performed to discriminate lectin receptor distribution in isolated single cell suspensions by double immunostaining.

Materials and Methods

Tissues. Twelve tonsils with reactive hyperplasia were obtained fresh from tonsillectomy patients after surgical extirpation. All specimens were divided into 4 parts; each part was fixed in one of 4 solutions: 10% nonbuffered formalin overnight, 10% buffered formalin for 4-6h, 95% ethanol overnight, or rapidly frozen. The frozen sections were air dried for 15min, fixed in acetone for 10min at room temperature, and stored at −20°C when not used immediately. Others were paraffin-embedded after fixation.

Reagents. All biotin- or fluorescein isothiocyanate (FITC)-cojugated lectins (Table 1) were purchased from E-Y Laboratories, Inc. (CA, USA). Phycoerythrin (PE)-conjugated Leu-4 (CD_3) was obtained from Becton Dickinson (CA, USA), and anti-human immunoglobulin (āIg) came from Tago Inc. (CA, USA). Mouse liver powder was obtained from Rockland (CA, USA), and Trypsin was from Difco Laboratories (Mich, USA). Avidin-biotin-peroxidase complex (ABC) was purchased from Bio Genex Laboratories (CA, USA), and diaminobenzidine tetrachloride was from Dojindo (Tokyo, Japan).

Staining procedures. Paraffin-embedded tissue sections were first deparaffinized with xylene, processed routinely and then treated with methanol-H_2O_2 to block endogeneous peroxidase activity. After digestion with 0.1% trypsin for 30min, they were stained using the ABC method as previously described (9, 10). Briefly, phosphate-buffered saline (PBS) (pH7.4) containing mouse liver powder (100μg/ml) was applied to the sections for 10 min to reduce the nonspecific background staining. The sections were treated with biotin-labeled lectins (15μg/ml) for 1h, then with ABC for 30min. Each step was followed by 3 washes with PBS. The color was developed with diaminobenzidine and H_2O_2. After counterstaining with Mayer's hematoxylin or methyl green, the sections were dehydrated and mounted. Controls for biotin-labeled lectins were performed as described previously (4).

Flow cytometric analysis. Cells were prepared from tonsils by mechanical disintegration and suspended in RPMI 1640 supplemented with 1% bovine serum albumin and 0.1% sodium azide at a density of 10^7 cells/ml. The cell suspension (50μl) was mixed with 25μl FITC-conjugated lectins (1mg/ml), then incubated with 25μl of PE-conjugated Leu-4 (1:1) or āIg (1:30 dilution) for 45 min at 4°C. The cells were washed once with fetal calf serum and once with RPMI 1640. Dual parameter analyses were performed using an Epics 753 (Coulter Electronics, Inc., Hiateah FL, USA).

Table 1 Lectins used in this study

Common name	Sugar specificity	Source
I. Glucose/mannose		
Con-A	αMan>αGlc	Concanavalin
LcH	αMan>αGlc	Lens culinaris
PSA	αMan>αGlc	Pisum sativum
II. L-Fucose group		
Lotus	αL-Fuc	Lotus tetragonolobus
III. N-acetylglucosamine group		
WGA	GlcNAc1-2>βGlcNAc	Triticum vulgaris
IV. N-acetylgalactosamine/ galactose group		
RCA-1	βGal>αGal	Ricinus communis
LBA	αGalNAc	Phaseolus lunatus
DBA	α-D-GalNac	Dolichos biflorus
PNA	Galβ, 1, 3GalNAc	Arachis hypogaea
SBA	α and β-D-GalNAc	Glycine maximus
SJA	β-D-GalNAC	Sophora japonica
MPA	αGalNAc>αGal	Maclura pomifera
PHA	D-GalNAc	Phaseolus vulgaris
BPA	GalNac>Gal	Bauhinia purpurea
GSA-1	D-Gal	Griffonia simplicifolia
V. Sialic acid group		
LPA	NeuNAc	Limulus polyphemus

Results

Histochemistry. First, we examined the influence of fixatives on lectin-binding patterns using WGA, PNA, SJA, and GSA-1 (Table 2). These lectins bound to all lymphocytes, but the binding intensity and patterns were fixative dependent. In frozen sections, the cellular morphology was poorly preserved, and the reaction was hardly detectable on most lymphocytes except on germinal center lymphocytes by PNA. Adequate morphological preservation was provided by nonbuffered and buffered formalin and ethanol fixation. However, the lectin binding to lymphocytes was poor in both buffered and nonbuffered formalin fixation. Nonbuffered formalin imparted nonspecific background staining that obscured the positive reactivity and sometimes the background staining was so

Table 2 The influence of fixatives on lectin binding patterns

Lectins	Fixatives	GC lymph	MZ lymph	PC lymph	Mac	Endo	S.E.
WGA	Acetone	−	−	−	++	++	++
	Non-buffered formalin	−	−	−	++	++	++
	Buffered formalin	+p	+p	+p	++	++	++
	Ethanol	++	++	++	++	+++	++
PNA	Acetone	+++	−	+p	+++	++	++
	Non-buffered formalin	−	−	−	+++	++	+
	Buffered formalin	+++	−	+p	+++	+++	++
	Ethanol	+++	+	+p	+++	+++	++
SJA	Acetone	−	−	−	+p	−	−
	Non-buffered formalin	−	−	−	−	−	−
	Buffered formalin	−	−	−	+p	+	−
	Ethanol	+	+	+	+	+	+
GSA-1	Acetone	−	−	−	++	−	−
	Non-buffered formalin	−	−	−	+	−	−
	Buffered formalin	−	−	−	+	−	++
	Ethanol	++	++	++	++	++	++

GC lymph: Germinal center lymphocytes; MZ lymph: Mantle zone lymphocytes; PC lymph: Paracortical lymphocytes; Mac: Macrophages/histiocytes; Endo: Vascular endothelial cells; S.E: Surface epithelium; +++: strongly positive; ++: moderately positive; +: weakly positive; +p: weakly and partially positive; −: negative.

Table 3 Lectin binding patterns in the tonsil

	Group A	Group B	Group C	Group D
Germinal center lymphocytes	+	+	−	−
Mantle zone lymphocytes	+	−	−	−
Paracortical lymphocytes	+	−	−	−
Macrophages/histiocytes	+	+	+	−
Endothelia	+	+	+	−
Surface epithelia	+	+	+	−

Group A: Con-A, RCA-1, LcH, WGA, MPA, PHA, PSA, PNA, SJA, GSA-1; Group B: BPA, Lotus; Group C: SBA, DBA; Group D: LBA, LPA.

strong that it was difficult to interpret the results. The strongest staining was obtained from ethanol fixation which yielded minimal background reactivity. The evaluations of lectin binding patterns reported here were based on specimens fixed with ethanol. We could not find any difference in the reactivity between trypsinized and nontrypsinized sections, and no correlation was found between the reactions and blood group status.

Lectins could be divided into four groups according to the binding affinity to different tissue components (Table 3): Group A (Con-A, RCA-1, LcH, WGA, MPA, PHA, PSA, PNA, SJA and GSA-1), binding to all tissue components; group B (Lotus and BPA), binding to germinal center lymphocytes and other tissue components with variable reactivity but not to mantle zone and paracortical lymphocytes; group C (SBA and DBA), nonreacting with lymphoid cells, but binding to other tissue components; group D (LBA and LPA), nonreacting with all tissue components. In macrophages/histiocytes and plasma cells, the reaction products were confined to the cytoplasm, whereas in lymphoid cells, they were confined only to the cell surface or seen both in the cytoplasm and on the cell surface. Cap-like staining confined to one pole of the cell membrane was not observed.

Among group A lectins, Con-A, RCA-1 and LcH reacted strongly with all lymphocytes (Fig. 1), and WGA, PHA and MPA reacted with moderate intensity. Other lectins except PNA reacted weakly. PNA reacted strongly with germinal center lymphocytes, weakly with mantle zone, and weakly and partially with paracortical lymphocytes (Fig. 2). Only Con-A and PNA reacted strongly and other lectins were moderately reactive to the macrophages/histiocytes. Vascular endothelium was intensely reactive with PNA (Fig. 3) and only moderately with other lectins. Surface and crypt epithelium of the tonsils were moderately reactive with all lectins except for PNA, which showed strong reaction.

BPA of group B lectins reacted moderately with lymphocytes and strongly with macrophages/histiocytes (Fig. 4). It also showed uniformly moderate reaction with vascular endothelium and surface epithelium. In contrast, the definable reactivity of Lotus was weaker than that of BPA.

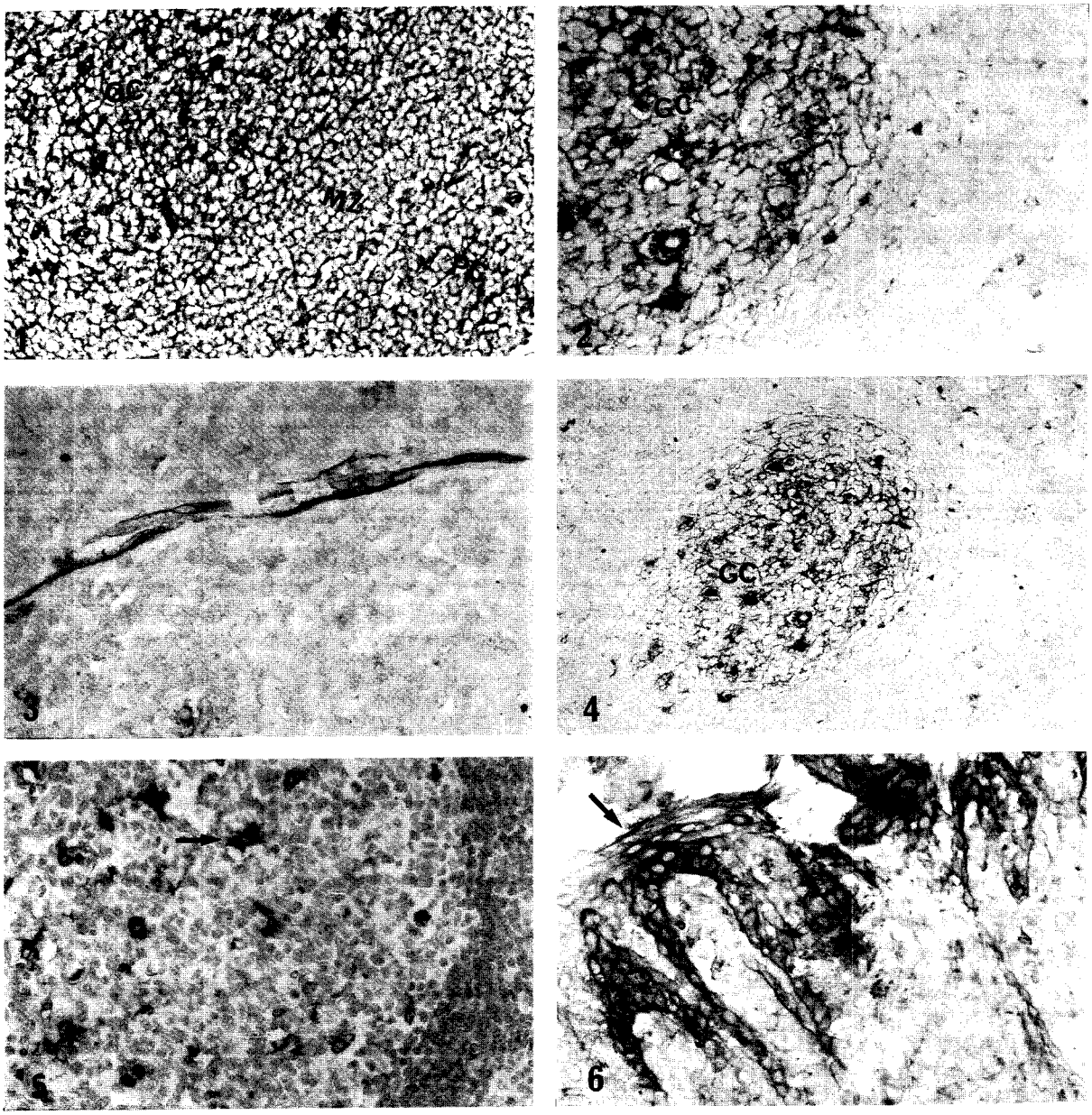

Fig. 1 LcH: All tissue components are strongly stained. GC: Germinal center. MZ: Mantle zone. PC: Paracortex. X400.
Fig. 2 PNA: Note strongly reactive germinal center lymphocytes (GC: Germinal center). X400.
Fig. 3 PNA: Note strong reaction for vascular endothelium. X400.
Fig. 4 BPA: Note the strong staining of macrophages and follicular dendritic cells and weakly reactive germinal center lymphocytes (GC: Germinal center). X200.
Fig. 5 SBA: Intense binding to tingible body macrophages (arrow) is noted. Lymphocytes are unstained. X400.
Fig. 6 DBA: Note strong binding to surface epithelial cells (arrow) sparing lymphocytes. X400.

Table 4 Percentage of lectin-binding lymphocytes in the T or B lymphocyte population of tonsils

Lectins	% of lectin binding cells in CD3+ cells	% of lectin binding cells in Ig+ cells
Con-A	98.5 ± 1.4	98.9 ± 1.0
RCA-1	98.9 ± 1.0	98.8 ± 1.0
LcH	93.8 ± 1.5	98.8 ± 0.5
WGA	99.7 ± 0.2	99.5 ± 0.4
MPA	99.4 ± 0.3	99.5 ± 0.5
PHA	99.8 ± 0.1	99.5 ± 0.5
PSA	92.5 ± 2.5	98.0 ± 2.0
PNA	20.4 ± 1.6	20.6 ± 1.5
SJA	49.5 ± 1.9	70.5 ± 0.5
GSA-1	6.3 ± 0.2	15.6 ± 2.0
BPA	27.5 ± 1.6	60.5 ± 0.5
Lotus	0.0	25.3 ± 0.7
SBA	94.5 ± 1.9	87.5 ± 0.4
DBA	0.0	0.0
LBA	0.0	0.0
LPA	0.1	0.0

Results are the average of three successive experiments with standard deviation.

SBA of group C lectins showed a uniformly strong reaction pattern with macrophages/histiocytes (Fig. 5), vascular endothelium and surface epithelium. DBA showed strong reaction with vascular endothelium and surface epithelium (Fig. 6), but weak reaction with macrophages/histiocytes.

Flow cytometry. The percentage distribution of lectin reactive lymphocytes is presented in Table 4. Thirteen of the 16 lectins bound to the lymphocyte cell surface with variable reactivity. DBA, LBA and LPA did not bind to the lymphocytes Lotus was exclusively expressed on some B-lymphocytes.

Discussion

Significant differences in the spatial distribution and intensity of lectin binding were observed among the different fixatives. Histochemically demonstrated lectin binding was more intense in sections fixed with ethanol. It was probable that ethanol fixed glycoconjugates more effectively *in situ* or preserved well their capacity to bind lectins better than other fixatives. These interpretations appear to contradict the findings of Wirbel *et al.* (6) who investigated the influence of several fixatives upon various lectin affinities and postulated the total destruction of affinity by ethanol fixation. However, a direct comparison is difficult because they used cryostat sections fixed with ethanol, but our study was done on tissues fixed with ethanol and then paraffin embedded. Differences between the two techniques for the processing of the tissue may cause masking of some reaction groups and unmasking of new groups. One study has attributed an overall weakening of the binding to processing with wax (11). The present study found that wax processing does not necessarily lead to the weakening of lectin binding, but the weakening is due to the primary fixing agents. Moreover, our study suggests that ethanol preserves lectin affinity better than other fixatives despite being processed through wax. In this regard, our study is in accord with Allison's report (7) which noted the superiority of ethanol as a fixative in lectin histochemistry. Ethanol fixation causes no crosslinking between protein chains, and the membrane of ethanol fixed cells is relatively mobile (12). This may leave more sites exposed in the glycoconjugate to accomodate binding. Ethanol fixation was superior to frozen sections both in the staining intensity and preservation of tissue architectures, suggesting that some carbohydrates may be lost from tissues if the associated protein is not immobilized.

Among group A lectins, the reactive patterns of Con-A, RCA-1, LcH, WGA, PHA, and PSA with lymphoreticular tissues corresponded to those described by Hsu and Ree (4), but the patterns of PNA and SJA were not consistent with their results which showed nonreaction with lymphoid cells. Strauchen (13) stated that at low concentration (10 μg/ml), Con-A reacted with only macrophages/histiocytes but at higher concentration (100-1000 μg/ml) with all lymphoid cells. Our study showed that Con-A bound to all lymphoreticular tissues at low concentration (15 μg/ml) as described by others (4, 14). LcH is a T-cell mitogen in mice (15), and shares a common binding receptor with Con-A (16, 17). Therefore it is likely to show a reaction profile similar to Con-A. LcH reacts with all lymphocytes from human peripheral blood (18, 19). Our cell suspension study from tonsils also showed all lymphocytes to be reactive. Macrophages/histiocytes were moderately reactive with Con-A in our study which contradicts the finding of Hsu and Ree (4) where they found very weak or no reaction. Although WGA has a sugar specificity that differs from that of Con-A, the reaction profile of WGA resembled that of Con-A. PHA, like Con-A, is a potent T-cell mitogen. However, PHA also stimulates B lymphocytes (20).

Some authors believe that PHA from various sources may have different properties (21). Concerning the reactivity of PHA with lymphoid cells and macrophages-histiocytes, our data are also in agreement with Wirbel *et al.* (6). Unlike Wirbel *et al.* (6), we found that all preparations reacted with vascular endothelium. Because the carbohydrate-binding specificity of PSA is similar to that of LcH (22), it is expected to have a similar binding profile (4). This was confirmed by our study. MPA lectin differs from Con-A, LcH, and PSA in its sugar specificity, but they all had similar staining profiles. In this context, Fowlkes *et al.* (23) reported no correlation between the complexity of lectin reaction on thymocytes and the nominal sugar specificities. SJA has a nominal specificity for α-D-N-acetylgalactosamine and is known to react with blood group B erythrocytes (24). In our study, however, SJA bound to vascular endothelium irrespective of blood group. PNA binds to human germinal center lymphocytes (2, 3, 13, 25), immature thymocytes (26), and a small population of tonsil T lymphocytes (26). In the present study, germinal center lymphocytes strongly reacted with PNA, but weak reaction was also observed on substantial number of mantle zone and paracortical lymphocytes.

Strong binding of PNA with macrophages/histiocytes as described by Hsu and Ree (4) was confirmed by the present study. According to Wirbel *et al.* (6), PNA binds only to capillary endothelium but not to large vessels. In our study, all vascular endothelium of the tonsil was reactive irrespective of their caliber. They did not find crypt epithelium of the tonsil to be reactive either, but we found an intense reaction, which is in agreement with Strauchen (13).

Selective binding of Lotus to germinal center lymphocytes (13) was confirmed by our study. BPA was reactive with germinal center lymphocytes in either frozen or ethanol-fixed sections but did not react at all in formalin fixation.

Though it has been shown that SBA binds to germinal center lymphocytes in frozen and paraffin sections of tonsil and lymph nodes (6, 13), we found no reaction with lymphocytes as reported by Hsu and Ree (4). It is of note that in cell suspensions of unfixed materials, SBA reacted with both B (93.6%) and T (87.2%) lymphocytes. The vulnerability of SBA binding site on lymphocotes to fixatives may explain these reported discrepancies and the difference between immunohistochemical and cell suspension studies. There are also discrepancies about SBA reactivity with macrophages/histiocytes (4, 6).

DBA consistently recognized vascular endothelium in a variety of human lymphoreticular tissues. This binding was irrespective of the blood group status of the tissue donors, which contradicts the results of Hsu and Ree (4) showing specificity for blood group A individuals. DBA binds to mouse immature thymocytes and a small population of bone marrow and splenic cells (27). In humans, we did not find any reactivity with lymphoid cells in either the immunohistochemical or flow cytometric study.

LPA agglutinates mouse helper T cells (15). The present study using human tissues demonstrated that LPA was nonreactive with all tissue components including lymphoid cells either by immunohistochemistry or by flow cytometric study.

In conclusion, the lectin binding pattern and intensity depends on the fixative used, and should be considered carefully in the selection of suitable fixatives for lectins under study. Lectins with dissimilar sugar specificities not unlikely show similar reaction pattern, and the reverse is true for lectins having similar sugar specificities. Reported discrepancies of the lectin binding pattern may be explained by the differences of tissue processing method including fixatives, sources of lectins, and species examined. The vulnerability of SBA receptors to the fixative was confirmed by flow cytometric analysis.

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Received August 3, 1992; accepted September 18, 1992.