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Preliminary characterization and distribution of vicia villosa binding cells in human tonsils

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Summary. The lectin Vicia villosa (VV) has been used for the separation of human and murine contrasuppressor T cells. These cells were characterized in cryostat sections of human palatine tonsils by double staining with VV lectin and monoclonal antibodies to macrophages, lymphocytes and their subsets using a fluorescein-rhodamine technique. VV lectin had an affinity for the $CD8^+$ subset of lymphocytes and for a subset of macrophages within the germinal centre. The number and distribution of VV lectin binding cells was studied in paraffin sections of formalin fixed tonsils by the avidin-biotin-peroxidase technique. Positive cells in the germinal centres, mantle, interfollicular zones and fibrous connective tissue septa were quantified using an image analyser. These were found in greatest density in the interfollicular zone, correlating with the known distribution of T cells in human palatine tonsils. The binding of VV lectin to a subset of macrophages appears not to have previously been described nor have VV lectin binding $CD8^+$ lymphocytes been demonstrated in sections of human tissues.

Key words: Tonsils – Vicia villosa lectin – Lymphocyte subpopulations – Contrasuppressor cells – Double labelling

Introduction

The lectin Vicia villosa (VV) was first used for selective affinity fractionation of murine cytotoxic T lymphocytes on the basis of its binding to a distinguishing surface glycoprotein, T145 (Kimura et al. 1979).

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Subsequent studies, however, demonstrated that VV lectin was not an exclusive differential marker for cytotoxic T lymphocytes (Conzelmann et al. 1980; Kaufmann et al. 1981; MacDonald et al. 1981). It does not distinguish between cytotoxic and suppressor T cells and the receptor for VV is expressed on blast cells generated from mixed leucocyte culture and from polyclonal activation of T cells (Lang et al. 1982).

Recent studies have shown that VV lectin binds preferentially to a subset of T cells and it has been used for the separation of human (Lehner et al. 1985) and murine (Green et al. 1981) contrasuppressor cells. In the present study direct staining of cells with an affinity for VV lectin was undertaken using biotinylated VV lectin on sections of human palatine tonsils. These were then subjected to quantitative analysis. Further characterization was done by double immunoenzymatic labelling using fluoresceinated VV lectin and a panel of monoclonal antibodies to macrophages, lymphocytes and their subsets which were visualised using tetramethylrhodamine isothiocyanate (TRITC).

Materials and methods

Ten human palatine tonsils obtained from patients operated for recurrent tonsillitis either at St. Bartholomew's hospital or Guy's Hospital were studied.

Immediately after tonsillectomy, aliquots of tissue were quick frozen in liquid nitrogen and stored at −190° C until sectioned. The remaining tissue was fixed in 10% formol saline pH 7.4 and subsequently dehydrated and embedded in paraffin wax.

Preliminary characterization of VV binding cells. Characterization of the VV lectin positive cells was done by a sequential double labelling-technique on air-dried 5 μm cryostat sections fixed in acetone at 4° C for ten min. Fluoresceinated VV lectin was applied for forty five min at room temperature. After thorough washing with phosphate buffered saline one of the follow-

Table 1. Specificity, working dilution and source of monoclonal antibodies used in the present study

Antibody	Specificity	Dilution	Source
UCHLI	T-cells	1:100	Dako Ltd
MB2	B-cells	1:5	Euro-diagnostics
EBM II	Macrophages	1:20	Dako Ltd.
Anti-CD8[a]	Suppressor/ Cytotoxic	Neat	Immunology department UMDS
Anti-CD4[a]	Helper/ Inducer	Neat	Guy's Hospital

[a] Ascites fluid of cell lines OKT8 (anti-CD8) and OKT4 (anti-CD4) grown in (CBA × BALB/C) F_1 mice

ing mouse monoclonal antibodies was applied for sixty min at room temperature; UCHLI (T cells), MB2 (B cells), EBM11 (macrophages), anti-CD8 and anti-CD4 in previously determined optimal dilutions (see Table 1). This was followed by incubation with TRITC labelled rabbit-anti-mouse antibody after routine washing.

These slides were scanned using an Olympus BH2 microscope with a RFL fluorescence attachment and a model PM-IOADS Olympus automatic photomicrographic system.

An HBO 100 watt super pressure Mercury lamp was used with a BP-490 filter and a DM-500+0.515 Dichroic mirror for blue excitation. A BP-545 filter and DM 580+0.590 Dichroic mirror were used for green excitation.

Each slide was initially scanned with the blue filter in situ for fluorescein excitation using a ×10 eyepiece and an Olympus ×20 objective. Cells fluorescing with the VV lectin were then viewed with the green filter in situ and a semi-quantitative estimation made of the cells showing double fluorescence with both the fluoresceinated VV lectin and one of the panel of monoclonal antibodies with TRITC.

Quantification of VV binding cells. Sections of fixed tissue were cut at 4 μm and stained with haematoxylin and eosin to confirm the diagnosis of chronic tonsillitis. Formalin-fixed, paraffin-embedded sections of the same thickness were also stained by the indirect avidin-biotin-peroxidase complex technique using biotinylated VV lectin (Sigma) as the primary reagent. Paraffin sections were dewaxed and incubated in 0.1% trypsin (Type II crude-porcine pancreas from Sigma) in Tris buffered saline pH 7.6 for sixty s at room temperature. A routine avidin-biotin peroxidase (ABC) technique was then used, with a 1/20 dilution of the lectin applied for sixty min at room temperature and DAB as substrate. Three sets of control sections were done, one without the primary (VV) layer, the second without the avidin-biotin peroxidase and the third with VV lectin which had been pre-incubated with N-acetyl-D-galactosamine. None of these showed any positivity.

Sections stained with VV lectin were scanned by an individual observer using a Zeiss MOP-videoplan image analysis system with a digitising tablet.

Four zones of tonsil were defined; germinal centre, mantle, interfollicular and fibrous connective tissue septa. In each tonsil ten of each type of zone were randomly selected, the areas measured and the number of VV lectin stained cells in these areas counted using a Zeiss ×10 objective and a ×10 eyepiece. Further, in each of these ten regions, the total number of cells in an area 100 μ^2 was counted in five randomly selected fields. This was not done in the connective tissue which was relatively acellular. Based on these observations the number of VV lectin positive cells per ten square millimeters was calculated for each of the four defined zones and the number of VV lectin positive cells per 1 000 000 cells for the first three areas defined.

This data was subjected to an analysis of variance on a "within-subject" basis.

Results

Cells with an affinity for VV lectin formed two morphologically distinct populations and this distinction was confirmed on double staining. One population was lymphocytes with abundant cytoplasm which showed heavy granular cytoplasmic positivity for VV lectin and a coarse pattern of nuclear chromatin (see Fig. 1a). The other population was macrophages with a distinct nuclear membrane and a diffuse, less intense pattern of cytoplasmic positivity for VV lectin (see Fig. 1b).

Preliminary characterization of VV lectin binding cells

On sequential double staining with fluoresceinated VV lectin and the panel of monoclonal antibodies the VV lectin binding cells in the germinal centres were identified as macrophages (see Fig. 2a and b). These cells were not labelled by any of the other antibodies. Outside the germinal centre, the VV lectin cells labelled with UCHLI and anti-CD8. A semi-quantitative analysis of this double labelling is shown in Table 2.

None of the VV lectin positive cells showed any labelling with MB2, the B-cell marker. Using immunofluorescence it was possible to distinguish the germinal centre of the tonsil but not accurately define the mantle and interfollicular zones, which were therefore designated extra-germinal centre (see Table 2).

Quantification of VV lectin binding cells

The distribution and density of the two morphologically distinct cell populations differed significantly in the four anatomical regions defined (see Table 3).

The VV lectin positive macrophages were seen exclusively in the germinal centre of the secondary follicles. No lectin binding lymphocytes were identified in this zone. VV lectin positive lymphocytes were present in the interfollicular zone and fibrous connective tissue septa in greatest density and in the fibrous connective tissue septa the density was greater in the more chronically inflamed tonsils.

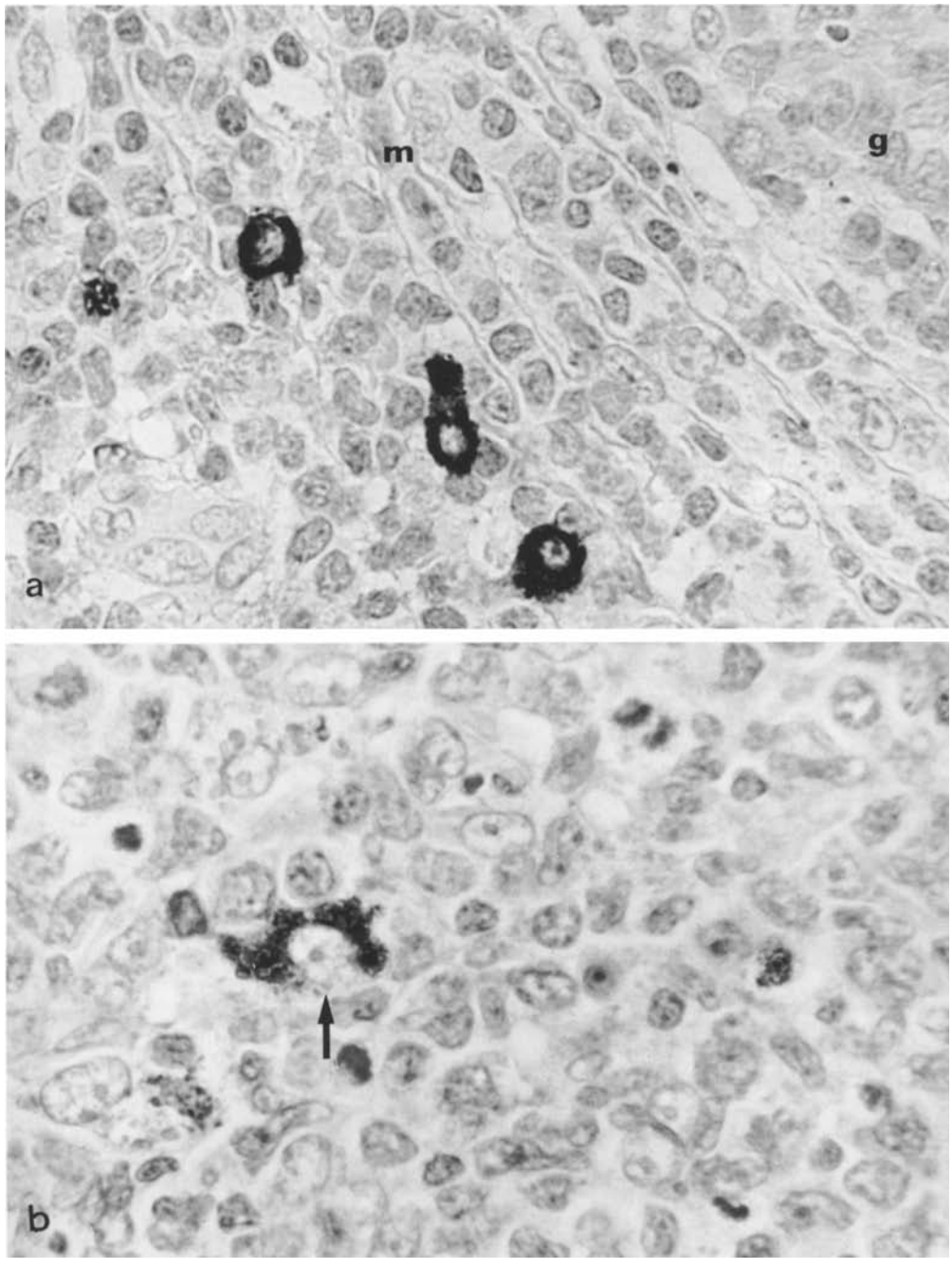

Fig. 1 a, b. Avidin-biotin-peroxidase staining for VV lectin, counterstained with Mayer's Haemalum. **a** Lymphocytes in the mantle zone (*m*) showing coarse granular cytoplasmic positivity (×800) (*g* = germinal centre). **b** Macrophage in the germinal centre with diffuse less intense cytoplasmic staining (×800)

They were also present in smaller numbers in the mantle region and within the epithelium.

The density of cells binding VV in the interfollicular area and the connective tissue was very similar. There were, however, significantly fewer VV positive cells in both the mantle and germinal centre region ($p < 0.001$) (see Table 3).

Discussion

The lectin Vicia villosa which has a high affinity for the sugar L-D-N-acetyl-galactosamine (Kaladas et al. 1981) has been used to separate a subset of $T8^+$ cells which has been postulated to function as contrasuppressor cells, presenting antigen to in-

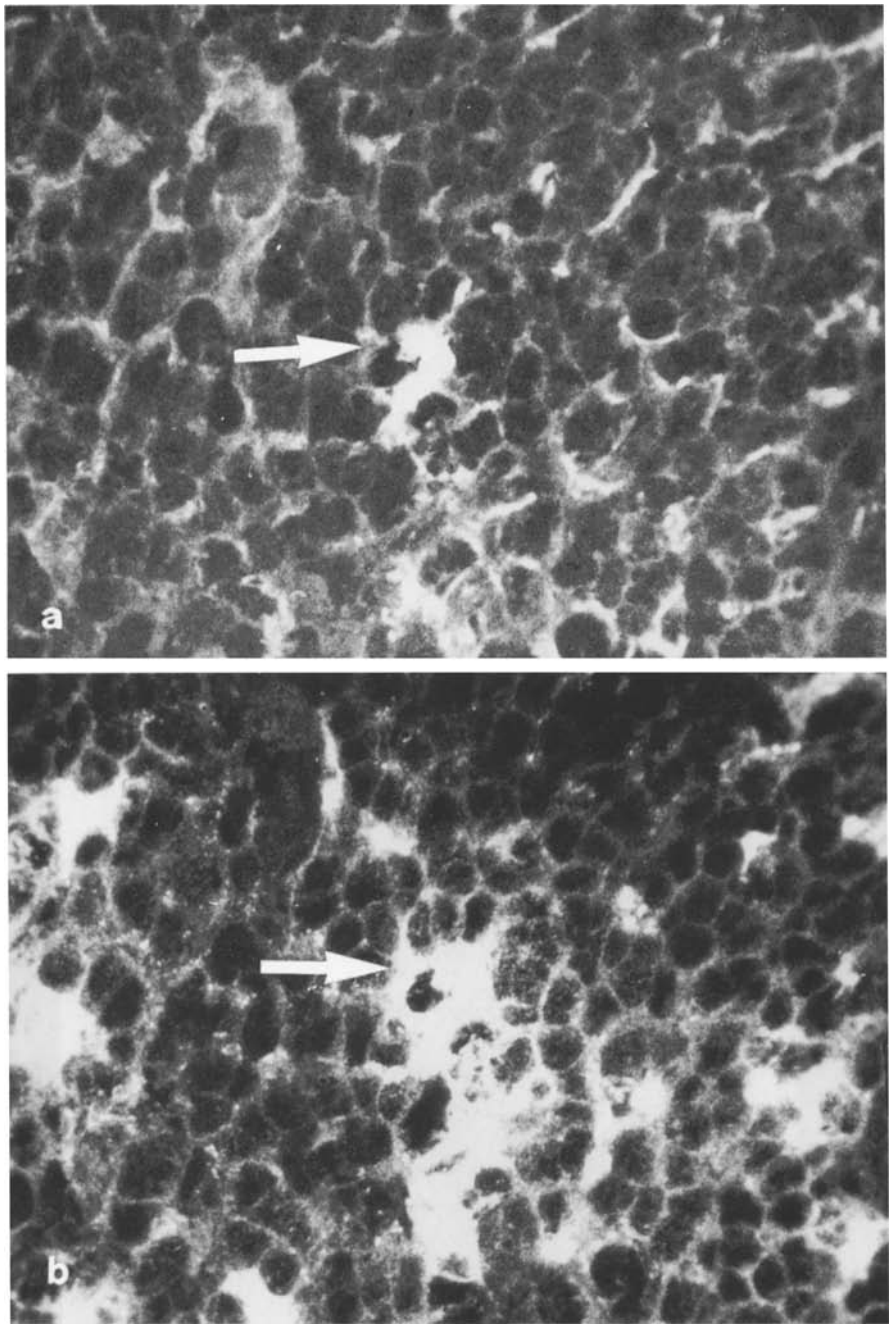

Fig. 2a, b. Double immunofluorescent labelling without counterstain.
a Cell in germinal centre stained with fluoresceinated VV lectin (× 500).
b Same cell stained with the macrophage marker (EBM 11) and rabbit anti-mouse tetramethylrhodamine isothiocyanate (× 500)

Table 2. Phenotypic characterization of VV lectin positive cells

VV lectin positive cells	EBM11 (Macrophages)	UCHLI (T Cells)	Anti-CD8 (T-suppressor)	Anti-CD4 (T-helper)	MB2 (B-cells)
Germinal centre	+	−	−	−	−
Extra-germinal centre	−	++	++	−*	−

* Two $CD4^+$ VV^+ cells were detected in 8 sections. Sections of tonsil were stained using sequential, fluorescent double-labelling with fluoresceinated VV lectin, mouse monoclonal antibodies to lymphocytes and macrophages and rhodamine labelled rabbit anti-mouse antibodies; − = none positive; + = occasional cells positive; ++ = moderate number of cells positive

Table 3. Distribution of VV binding lymphocytes and macrophages

Zone	Number per ten square millimetres (mean ± SD)			Number per 1,000,000 cells
	Moderate chronic inflammation (7)	Marked chronic inflammation (3)	Total (10)	(Sample size 10)
Mantle (Lymphocytes)	116 ± 10	124 ± 12	119 ± 11	54 ± 5
Interfollicular (Lymphocytes)	630 ± 34	626 ± 20	629 ± 29*	402 ± 19
Germinal centre (Macrophages)	60 ± 13	52 ± 3.6	58 ± 12**	52 ± 10
Fibrous connective tissue (Lymphocytes)	512 ± 28	1040 ± 26***	670 ± 257	N.D.

* The numbers in the interfollicular region are significantly greater ($p < 0.001$) than in the mantle or germinal centre but similar to those in the fibrous connective tissue septa

** The numbers in the germinal centre are significantly less than in any other region ($p < 0.001$)

*** The numbers in the fibrous connective tissue septa of the more chronically inflamed tonsils are significantly greater ($p < 0.001$) than in those with moderate chronic inflammation

N.D. Not determined, as the fibrous connective tissue septa are relatively acellular

duce helper activity and prevent suppressor activity (Lehner et al. 1985). This cell may have considerable biological significance and has not previously been demonstrated in sections of human tissue. In this study VV lectin binding lymphocytes have been demonstrated in human tonsils where, in the areas of highest density they constitute 0.04% of the total cell population; $CD8^+$ VV lectin positive lymphocytes are a subpopulation of these. The distribution of the VV positive lymphocytes, with the bulk of the population in the interfollicular zones, is consistent with the known distribution of T lymphocytes in human tonsils (Berling et al. 1986). However, a higher density of VV lectin positive lymphocytes was seen in the fibrous connective tissue septa of the more chronically inflamed tonsils, the significance of which is not apparent.

The data presented in this study, using sequential double immunofluorescent labelling, agree with the findings of other workers in that VV lectin does not have an affinity for B cells (Lang et al. 1982) and appears to bind preferentially to the $CD8^+$ subset of T cells (Lehner et al. 1985). However, the affinity of VV lectin for a subset of macrophages appears not to have been previously reported and the functional significance of this cell population is yet to be determined. The exclusive presence of these cells in the germinal centre raised intriguing possibilities about their role in immunoregulation.

Experimental work in animals has suggested that contrasuppressor cells may play a significant role in vivo. These cells might be important in immunoregulation, allowing microenvironmental immune responses to take place while systemic immunity is suppressed (Green et al. 1982). Contrasuppression is thought to play an important role in tolerance (Iverson et al. 1983; Ptak et al. 1984; Suzuki et al. 1986) and it has been postulated that the balance of suppression and contrasuppression may determine tolerance to antigens (Ptak et al. 1984).

Animal models with both spontaneous (Kelly et al. 1987) and induced (Smith et al. 1982; Smith et al. 1983) autoimmune disease have been used to demonstrate that abnormal contrasuppressor function is associated with the development of autoimmune disease (Smith et al. 1982; Smith et al. 1983; Kelly et al. 1987). It has also been suggested that contrasuppression might account for autoimmunity at the local level (as in autoimmune thyroiditis) despite systemic suppression (Lehner 1986). Graft versus host disease is more easily induced in neonatal mice whose spleen cells are proficient at developing into contrasuppressor cells (Green et al. 1981) and it may be that contrasuppression facilitates the development of graft versus host disease.

In the present study, VV lectin binding cells have been demonstrated in human tonsils and quantification of these cells in tissue sections from patients with systemic and local autoimmune diseases such as systemic lupus erythematosus and autoimmune thyroiditis and possibly in patients with graft versus host disease may have prognostic significance. The fact that VV lectin also has an affinity for a subset of macrophages should not be a drawback as the two populations of cells are morphologically distinct.

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