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Recognition of a CD4$^+$ mouse medullary thymocyte subpopulation by *Amaranthus leucocarpus* lectin

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SUMMARY

We have used the Galβ(1→3)GalNAc-specific *Amaranthus leucocarpus* lectin to isolate a thymus cell subpopulation which is different from that sorted with *Arachis hypogaea* lectin. The cells recognized by *A. leucocarpus* lectin were predominantly CD4$^+$, whereas a minor proportion of CD8$^+$ cells (approximately 11%) were also identified. The *A. leucocarpus*-positive cells were located in the thymus medulla and the cortico-medullary junction. The cortex was negative for *A. leucocarpus* cells.

INTRODUCTION

Lectins employed as mitogens are immunological tools for understanding the mechanisms of antigen-driven cell activation;[1,2] their mitogenic activity is not restricted to a specific saccharide structure as it has been demonstrated with phytohaemagglutinin (specific for complex oligosaccharides) nor concanavalin A (specific for α-D-mannose and α-D-glucose).[3,4] It has recently been shown that a Galβ(1→3)GalNAc-specific lectin isolated from the seeds of the jackfruit *Artocarpus heterophyllus*[5] is mitogenic for T lymphocytes,[6] as opposed to *Agaricus bisporus* and *Arachis hypogaea* lectins, both of which share the sugar specificity of *Artocarpus heterophyllus*.[7,8] We have recently described a new Galβ(1→3)GalNAc-specific lectin from *Amaranthus leucocarpus* (ALL)[9–11] which is not mitogenic for the T cells of healthy individuals. Since some of these lectins are widely used for separating mature from immature lymphocytes[6] and mouse thymocytes,[12] the aim of this work was to determine if the lectin from *A. leucocarpus* could differentiate a specific mouse thymocyte subpopulation.

MATERIALS AND METHODS

Reagents

Peanut agglutinin (PNA), dimethyl sulphoxide, pronase (*Streptomyces griseus*, Sigma fraction XXV), fluorescein isothiocyanate (FITC), tetramethyl rhodamine B isothiocyanate (TRITC), Hanks' solution, bovine serum albumin (BSA), nitro blue tetrazolium (NBT), dimethylformamide, neuraminidase type V, *N*-acetyl-D-Galactosamine, alkaline phosphatase-labelled avidin, peroxidase-labelled streptavidin, 3,3′ diamino benzidine tetrahydrochloride (DAB), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Sephadex G-50 were purchased from Sigma Chemicals (St Louis, MO). Isopentane, chloroform and acetone were from Merck, Mexico. Biotin-labelled as well as non-labelled rat monoclonal antibodies directed to the mouse CD4 (L3T4) and CD8 (Lyt-2) molecules as well as peroxidase-labelled rabbit anti-rat immunoglobulin G (IgG) were purchased from Pharmingen (San Diego, CA). Mouse dendritic cells were determined with rat monoclonal antibody NLDC-145.[13]

Lectin purification

Amaranthus leucocarpus seeds were obtained in Tulyehualco, Mexico. The lectin was purified by affinity chromatography on a column containing human type O red blood cell stroma as described by Zenteno & Ochoa.[10] The haemagglutination activity of *A. leucocarpus* (ALL) was tested against pronase-treated O human erythrocytes.[11] ALL (agglutinating activity of 1 : 15 000) was labelled with FITC, at a label to protein ratio of 3·5,[14,15] and with Immunopure *N*-hydroxysuccinimidobiotin from Pierce Chemical Co. (Rockford, IL) according to instructions.[16]

Preparation of thymus sections

Thymus gland was surgically removed from 3-week-old male BALB/c mice under anaesthesia. The specimens were divided into two parts. One was embedded in OCT (Tissue Tek) compound and immediately frozen in cold isopentane and the other fixed in 10% formalin and processed for light microscopy. Serial sections of 7-μm thickness were cut, on the same day, with a Bright FS/FCS cryostat from the frozen specimen, fixed in cold acetone/chloroform (v/v), air dried and kept frozen until used.

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Immunofluorescent and immunohistochemical staining of tissue sections

Fixed sections were washed extensively with phosphate-buffered saline, pH 7·2 (PBS) incubated for 15 min with 3% normal mouse serum and washed for 10 min with PBS before staining. FITC-labelled ALL was used at saturating concentrations as determined by previous titration experiments. Sections were incubated for 25 min at room temperature in a humidified atmosphere with 15-μl aliquots of the lectin. The sections were then washed for 30 min with PBS and mounted with PBS/glycerol before each slide was examined by two independent observers on a standard 14 Zeiss microscope equipped with an IV/2 epifluorescence condenser. Specificity controls were performed using non-conjugated lectin at the same protein concentration range as the first layer reagents. Slides treated in this way were always negative. Some experiments were performed with biotin-labelled lectin; in these experiments alkaline phosphatase-labelled avidin was used as second ligand and 5 μl/slide of NBT/BCIP[17] was used as substrate. Double stains were performed with tissue sections first stained with biotin-labelled ALL, as described above, and then incubated, separately, with unlabelled CD4 and/or CD8 rat monoclonal antibody for 15 min; as a second layer a 1 : 200 dilution of the peroxidase-labelled rabbit anti-rat IgG was used and DAB was used as a chromogen. In order to determine whether medullar stromal cells were macrophages or dendritic cells double stains were performed with the biotin-labelled ALL and NLDC-145 monoclonal antibody, as described above.

Fractionation of thymocytes

Surgically removed thymuses were minced in Hanks' solution with a 20-gauge needle in a Petri dish, washed thrice in PBS, counted and adjusted to 1×10^8 cells/ml in PBS. Similar to the method used for the isolation of PNA$^+$ and PNA$^-$ thymocytes,[12] 1 ml of the cell suspension was incubated with 100 μg of ALL in PBS for 1 hr at room temperature. Two hundred and fifty microlitres of this solution was poured onto a glass tube (10 × 1 cm) containing 7% BSA in PBS and left for 30 min at room temperature before collection of the supernatant and the precipitate. After separation, the cells were incubated with 0·2 M GalNAc for 30 min at room temperature, washed twice with PBS and adjusted to 2×10^6 cells/ml in PBS. To confirm the specificity of the lectin binding 100 μl of the ALL$^+$ and ALL$^-$ cell suspension were incubated, separately in 3 ml glass tubes, for 30 min at room temperature with the optimal concentration, as determined in previous titration experiments, of FITC-labelled ALL. In order to determine the percentage of CD4- and CD8-positive cells, the ALL$^+$ and ALL$^-$ isolated cells were incubated with biotin-labelled CD4 and CD8 monoclonal antibody, the peroxidase-labelled streptavidin was used as a second layer, and DAB was used as the chromogen. At the end of the incubation period, cells were washed twice in PBS, mounted in PBS/glycerol and observed by light microscopy. In some experiments the lectin-isolated cells were incubated with 0·005 IU/l of sialidase before performing the fluorescence assays.

FACS analysis

The amount of ALL-positive thymocytes was also determined in a Becton Dickinson FACScan using forward angle laser light scatter to exclude dead cells. ALL-stained thymocytes were resuspended in 0·5 ml of buffered 1% paraformaldehyde and 5000–10 000 cells were counted. The number of ALL-positive cells was determined in three different experiments using solely FITC-labelled lectin.

RESULTS

All the experiments in this study were performed using a subagglutinating dose of the lectin (1 μg/ml). The number of *Amaranthus leucocarpus*-positive thymocytes, determined in three independent experiments by the absolute number of aggregated cells and the percentage of fluorescent cells per microscopic field, corresponded to 7% of the total (roughly 150×10^6) cells. Neuraminidase treatment of the thymocytes showed a minimum increase in the amount of ALL-positive cells as opposed to PNA-positive cells which showed a significant increase. When thymic cells were first fractionated into PNA$^+$ and PNA$^-$ populations, less than 3% of the former reacted with ALL whereas none of the latter did. Seventy-five per cent (60%–87%) of the *A. leucocarpus*-positive thymocytes were CD4$^+$ and 11% (3%–15%) of the ALL$^+$ cells were CD8$^+$; these results represent the mean of 10 different experiments.

Immunohistochemical analysis showed that the ALL$^+$ cells were located in the thymus medulla, only a small percentage of positive cells were found in the cortico-medullary junction. The cortex was negative for ALL cells (Fig. 1). Immunofluorescence with FITC-labelled ALL suggested that a very small percentage of stromal cells were also ALL$^+$, therefore further analysis was performed with biotin-labelled lectin as well as with a dendritic cell-specific monoclonal antibody. Figure 2 shows ALL$^+$ stromal cells located only in the thymic medulla; this cell corresponded to a dendritic cell as determined by NLDC-145 monoclonal antibody stain. Double-staining analysis showed that ALL$^+$ dendritic cells interact mainly with some CD4$^+$, ALL$^-$ thymocytes since there was only one ALL$^+$ thymocyte near the dendritic cell (Fig. 3).

FACS analysis with FITC-labelled ALL showed that 5–7% of the thymus cells were positive, thus corroborating the percentage found in the immunofluorescence assays (Fig. 4).

DISCUSSION

One classification of thymocytes is based on the expression of the CD4 and CD8 markers; the immature double negative subset contains all of the cells with thymus-homing precursor potential whereas the single positive subsets are regarded as mature cells because they show the functional properties of peripheral T cells.[18] Nevertheless, thymocytes can also be classified by their ability to bind peanut agglutinin.[19] PNA$^+$ cells are considered immature despite the fact that they can possess the CD4$^-$,CD8$^-$ or CD4$^+$,CD8$^+$ phenotype. The latter are considered intermediates between progenitor cells and mature T cells.[20–22] Generally immature thymocytes are located in the thymic subcapsular zone and cortex whereas mature cells are found in the medulla.

In this study we have shown that the lectin isolated from *Amaranthus leucocarpus*,[11] which shares closely related sugar specificity with PNA, recognizes a different and more mature thymic cell population. Although 11% of the cells were CD8$^+$ the majority of the cells binding ALL had the CD4$^+$ phenotype

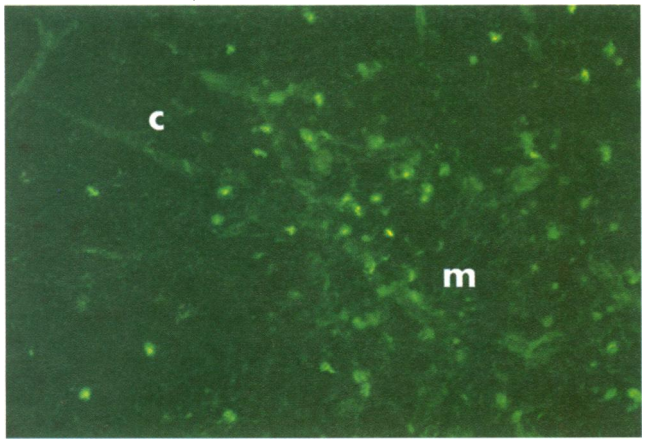

Figure 1. Photomicrograph (×31) of a longitudinal mouse thymus section stained with FITC-labelled *Amaranthus leucocarpus* lectin Positive cells were mainly located in the medulla (m) and cortico-medullary junction (c).

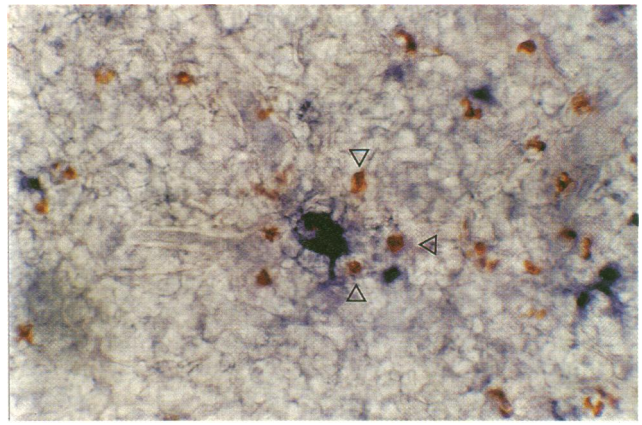

Figure 3. Photomicrograph (×55) of a double-stained longitudinal mouse thymus section showing a ALL$^+$ dendritic cell surrounded by CD4$^+$ thymocytes (arrows) and one *A. leucocarpus*-positive thymocyte (dark blue).

and were located in the medulla thus reinforcing the mature stage of this cell population. None of the ALL$^+$ cells were double negative. As shown in Figure 3 we have observed that some of the ALL$^+$ stromal cells were NLDC-145$^+$ cells and therefore they can be considered dendritic cells[23] which express among others adhesion molecules but do not express the Thy-1 nor the CD4 markers.[24] It is interesting to recall that thymic cells stained with NLDC-145$^+$ antibody correspond to non-lymphoid cells with dendritic appearance in the medulla and corticomedullar region.[13] The majority of thymocytes bound to the ALL$^+$ dendritic cells were of the CD4 phenotype and may be in the deletion process,[25] nevertheless, scarce ALL$^+$ thymocytes were located near dendritic cells suggesting that this cell subpopulation may correspond to stage III thymocytes[26] which have already gone through the negative selection process since the majority of ALL$^+$ peripheral blood lymphocytes are PNA$^-$ and either CD4$^+$ or CD8$^+$. Despite this, the biological role of the ALL$^+$, CD4$^+$ circulating lymphocytes remains obscure.

The discrepancy in the maturation stage of thymocytes isolated by PNA or by ALL may be explained by their different sugar specificity. PNA recognizes the terminal galactose of the Galβ(1→3)GalNAc disaccharide only when it is deficient in neuraminic acid;[27] PNA$^-$ cells treated with sialidase became PNA$^+$ thus suggesting that the PNA receptors on the mature cells are cryptic.[12,28] The combining site of the *A. leucocarpus* lectin accommodates the *N*-acetylgalactosamine residue found in the inner core of *O*-glycosidically linked glycans[9] independently of the presence or absence of neuraminic acid in the disaccharide, as our results with sialidase-treated thymocytes revealed. The lectin from *Artocarpus heterophyllus* which binds the Galβ(1→3)GalNAc disaccharide[29] recognizes mature T human peripheral blood cells;[6] nevertheless, its sugar specificity is controversial, some authors suggest that it recognizes the terminal galactose of the disaccharide[29,30] whereas Swamy *et al.*[31] affirm that its combining site is similar to that of *Amaranthus leucocarpus* lectin.

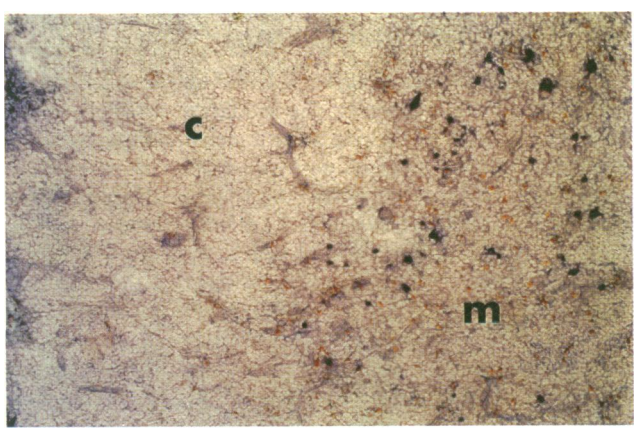

Figure 2. Photomicrograph (×13·75) of a double-stained longitudinal mouse thymus section. Biotin-labelled *Amaranthus leucocarpus*-positive cells (dark blue), and rat anti-mouse CD4$^+$ cells (light brown). Double-positive cells were only present in the medulla (m), whereas none was detected in the thymic cortex (c).

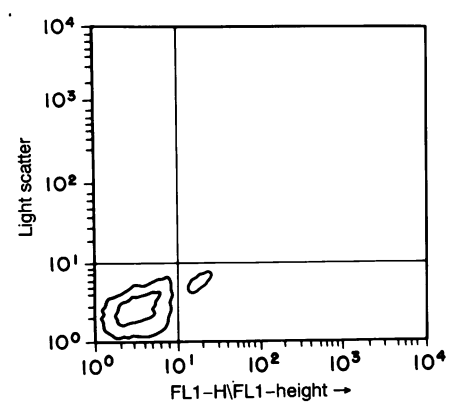

Figure 4. FACScan analysis of ALL-positive cells. Thymocytes from BALB/c mice were suspended in PBS, incubated with FITC-labelled lectin, washed and suspended in 1% paraformaldehyde. The amount of positive cells was 5–7% as determined in three different experiments.

The different cell populations recognized by lectins with apparently similar sugar specificity deserve a more profound analysis since there are many more surface markers which determine the biological role of a cell.

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