LYMPHOCYTE MEMBRANE LECTINS. DIRECT VISUALIZATION BY THE USE OF FLUORESCEINYL-GLYCOSYLATED CYTOCHEMICAL MARKERS

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1. Introduction

Lectins are known to bind selectively carbohydrate moieties and to be present as water-soluble proteins in a large variety of organisms [1–3]. Recently, it was found that lectins were also present as membrane components of various cells [4,5] and especially of mouse lymphocytes [6]. So far, membrane lectins have been mainly characterized by biochemical methods from isolated membranes. We describe here the direct visualization of membrane lectins *in situ*, by the use of fluoresceinyl derivatives of glycosylated cytochemical markers [7,8].

2. Materials and methods

Balb/c mice, ~6 weeks, were purchased from CSEAL Orléans-la-Source. Thymuses and spleens were teased in RPMI 1640 medium (Gibco, Grand Island Biol. Co, NY) and the cell suspensions were filtered through a nylon mesh in order to separate the cells from the undissociated material. The cells were then pelleted by centrifugation (150 × *g*, 10 min, at 4°C).

Mouse spleen cells were suspended in a red blood cell lysing buffer (0.015 M NH_4Cl, 0.02 M $KHCO_3$, 0.13×10^{-3} M EDTA, pH 7.4) for 4 min at 4°C. The cell suspension was then washed twice with phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate buffer (pH 7.4) PBS) containing 0.1% bovine serum albumin (Calbiochem Grade V) and the cells were spun down as above. For thymus cells, the lysing step was omitted. In all the experiments, the viability of the cells, tested by eosin exclusion, was > 90%.

2.1. *Glycosylated cytochemical markers*

Glycosylated cytochemical markers were prepared as in [7]. Bovine serum albumin (fraction V) and ferritin (Sigma Chemical, St Louis) [7] were used as carriers.

The *p*-nitrophenyl derivatives of β-lactose (Interchim, Montluçon), of α-D-mannopyranose and of α-L-fucose (Koch-Light Labs, Colnbrook), of β-di-*N*-acetylchitobiose [9] and of α-D-*N*-acetylgalactosamine [10] were reduced by catalytic hydrogenation. The aminophenyl derivatives were treated with nitrous acid, and the diazo derivatives were added to a solution of bovine serum albumin or of ferritin. The glycosylated proteins were purified by chromatography on an Ultrogel ACA 54 (Pharmindustrie, Clichy) then freeze dried. The sugar content of glycosylated bovine serum albumin was determined either by the orcinol sulfuric method [11] or by a spectrophotometric method. Glycosylated bovine serum albumin was dissolved in 0.15 M NaCl, 0.05 M sodium acetate buffer (pH 4.5). The spectra were scanned from 500–240 nm using the related buffer as a blank. The no. of azophenyl residues/mol protein was calculated by assuming mol. wt 64 000 for bovine serum albumin. The specific absorbance of azophenyl group was determined [12] by plotting the glycosylated serum albumin A_{350} versus the number of sugar

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residues per molecule of protein determined by the orcinol method.

2.2. *Fluoresceinyl glycosylated bovine serum albumin and ferritin*

Fluorescein isothiocyanate (Research Organics, Cleveland) was dissolved in freshly distilled dimethylformamide (20 mg/ml) and diluted (10 vol.) in pure ethylene glycol. The glycosylated protein (10 mg) was dissolved in a mixture of ethylene glycol (0.5 ml) and of 0.1 M sodium carbonate buffer (pH 9.3) (1.5 ml). The protein solution received the solution (0.5 ml) of the fluorescent reagent, and was stirred for 24 h at 4°C. Fluoresceinyl glycosylated proteins were purified by gel filtration on a column (2 × 50 cm) of Sephadex G-50 (Pharmacia, Uppsala) or Ultrogel ACA 54 (Pharmindustrie, Clichy). Fluoresceinyl glycosylated bovine serum albumins were further dialysed against distilled water for 24 h at 4°C and finally freeze dried. The number of fluorescent molecules bound per molecule of albumin was calculated from A_{495}/A_{280} [13]. Control fluorescent proteins were prepared using unmodified bovine serum albumin or ferritin.

Fluoresceinyl glycosylated bovine serum albumins were succinylated as described for wheat germ agglutinin [14]. The fluoresceinyl succinylated glycosylated albumins were purified by gel filtration on an Ultrogel ACA 54 column and then freeze dried. They were dissolved just before use, in order to avoid the leakage of the azophenyl sugars [15].

2.3. *Fluoresceinyl glycosylated marker labelling*

Mouse thymus and spleen cells (5×10^6 cells/ml) were suspended in phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and a fluorescent glycosylated marker (10–500 μg/ml); the cells were incubated for 5–60 min at 4°C. Then, cells were pelleted by centrifugation (150 × *g*, 10 min) at 4°C and washed twice with phosphate-buffered saline (pH 7.4) and mounted in 50% glycerol and observed by fluorescence microscopy in a Zeiss photomicroscope. Fluorescence was photographed with Tri-X-Panfilm.

Control experiments were conducted using:

(i) Fluoresceinyl thiocarbamyl ferritin or succinylated fluoresceinyl thiocarbamyl bovine serum albumin.

(ii) The fluorescent glycosylated markers in the presence of their related free sugars (0.3 M), or in the presence of the related non-fluorescent glycosylated marker (1 mg/ml).

3. Results and discussion

3.1. *Fluoresceinyl cytochemical markers*

Bovine serum albumin was used as a non-glycosylated macromolecular carrier of specific sugars and of the fluorescent label. The number of sugar units bound on glycosylated serum albumin was > 5/protein molecule (table 1). The ultraviolet spectra of the glycosylated cytochemical markers had a maximum intensity at A_{350} in contrast with the data in [12] with an A_{370} max. This discrepancy can be explained by the fact that the diazophenyl sugars react with a variety of amino acids, that the amino acid–azo conjugates have different intensity maxima and that the nature of the substituted amino acids depends upon the extent of the substitution [15].

The glycosylated proteins were assessed by double diffusion-technique versus various lectins. Precipitation bands were only obtained when the lectins obtained from Pharmindustrie (Clichy) were allowed to diffuse against the glycosylated protein bearing the specific related sugar: peanut agglutinin with lactosyl-markers, Con A with mannosyl-markers, *Ulex europaeus* agglutinin with fucosyl-markers, *Helix pomatia* agglutinin with GalNAc-markers and wheat germ agglutinin with GlcNAc–GlcNAc markers. These results are in agreement with the presence of several sugar units per mol protein and with the absence of unbound sugars.

Table 1
Number of sugar units/bovine serum albumin molecule

Markers	Sugar units/protein molecule	
	Orcinol reaction	Ultraviolet
BSA	0	0
CB-BSA	–	5.9
Fuc-BSA	–	5.6
GalNAc-BSA	–	5.7
Lac-BSA	5.4	5.4
Man-BSA	11	11

BSA, bovine serum albumin; CB, di-*N*-acetylchitobiose; Lac, lactose

Table 2
Absorbance data[c] of the succinylated fluoresceinyl glycosylated bovine serum albumins

Markers	$\frac{A^{495}}{A_P^{280}}$ [a]	$\frac{A^{495}}{A_P^{280}}$ [b]
BSA	1.15	3.25
CB-BSA	0.87	3.27
Fuc-BSA	0.75	2.90
α-GalNAc-BSA	0.57	2.94
Lac-BSA	0.52	2.85
Man-BSA	0.47	3.15

[a] Substitution in buffered saline
[b] Substitution in the presence of ethylene glycol
[c] $A_P^{280} = A^{280} - 0.33\,A^{495}$ [13]

The A^{495}/A_P^{280} ratio approximatively gives the number of fluorescein (ε_M = 73 000) molecules per molecule of bovine serum albumin ($A_{1\%}^{280}$ = 12)

The first attempt to prepare the fluoresceinyl derivatives of glycosylated proteins, using a buffered saline led to a low labeling level; the A_{495}/A_{280} was < 1. The labeling using fluoresceinyl isothiocyanate in the presence of ethylene glycol gave a high labeling. The minimum number of fluoresceinyl labels/protein molecule was ~3 (table 2). Using the fluoresceinyl derivative of bovine serum albumin, it was found that all the cells were at least slightly fluorescent. In order, to avoid this unspecific binding, fluoresceinyl bovine serum albumin and its glycosylated derivatives were succinylated. These fluorescent succinylated markers gave no evidence of unspecific labeling of the cells.

3.2. *Cell labeling*

The results obtained with the various glycosylated derivatives of bovine serum albumin are summarized in table 3. Consistently, identical results were obtained when the fluorescent derivatives of glycosylated ferritins (100 μg/ml) were used instead of those of bovine serum albumin. The labeling obtained with the glycosylated derivatives of ferritin could be prevented when cells were incubated with a fluorescent marker in the presence of the related free sugar (0.3 M) or in the presence of the related fluorescein-free glycosylated marker (1 mg/ml). The labeling obtained with the glycosylated derivatives of bovine serum albumin was greatly diminished but not totally abolished when cells

Table 3
Specific binding of succinylated fluoresceinyl glycosylated markers on murine spleen and thymus cells, in triplicate

Fluoresceinyl markers[a]	Spleen cells %	Thymus cells %
Suc-BSA	< 1	< 1
CB-Suc-BSA	10 ± 5	15 ± 5
Fuc-Suc-BSA	< 1	< 1
GalNAc-Suc-BSA	15 ± 5	10 ± 5
Lac-Suc-BSA	15 ± 5	30 ± 5
Man-Suc-BSA	25 ± 5	10 ± 5

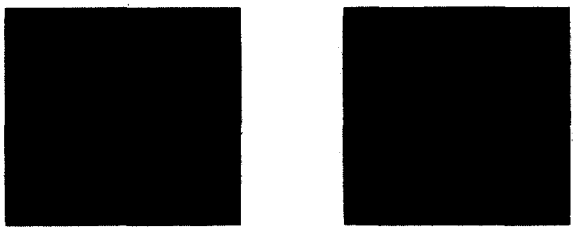
[a] Suc, succinylated; other abbreviations as under table 1

were treated in the presence of the related inhibitors. However, because the labeling intensity was greater when derivatives of bovine serum albumin were used instead of those of ferritin and because the number of specifically labeled cells were identical in both cases, all the further experiments were carried out using the derivatives of bovine serum albumin.

The number of labeled cells with the various markers was 10–25% with spleen cells and 10–30% with thymus cells. The number of cells labeled with certain markers in the case of spleen cells and in the case of thymus cells was significantly different.

Therefore, we may conclude that there are several sugar-binding proteins with different specificities. Indeed, if one sugar-binding lectin was able to bind various simple sugars as did the anticarbohydrate immunoglobulin [16] we would expect to have a similar number of cells labeled with the various glycosylated markers. The presence of several sugar-binding proteins on spleen and/or on thymus cells is in agreement with the data showing that the agglutination of a crude membrane extract was better inhibited by a mixture of simple sugars or of glycopeptides than by a unique sugar or glycopeptide [6].

The largest population of thymus cells bearing a sugar-binding protein was labeled with the lactosyl marker in agreement with results showing that lactose was able to elute the main agglutinating activity of thymocyte extracts adsorbed on glutaraldehyde-treated lymphocytes [6]. The largest population of spleen cells bearing a sugar-binding protein was labeled with a mannose-substituted marker in agreement with the results using mannose as an eluting



a b

Fig.1. The distribution of succinylated fluoresceinyl lactosyl-bovine serum albumin on thymus cells: (a) Cells were labeled for 30 min with the fluoresceinyl marker (10 μg/ml) at 4°C; (b) cells were labeled by a further incubation for 20 min at 37°C.

agent of splenocytes extracts adsorbed on glutaraldehyde-treated lymphocytes [6].

The % of cells bearing a sugar-binding protein is much higher than that expected to bear any antibody or antibody like molecule with a specific binding towards a sugar antigen. The presence of sugar-binding protein in both thymus and spleen cells supports also the idea that these sugar-binding proteins are not antibodies or antibody-like molecules.

When cells were incubated in the presence of a fluorescent glycosylated marker at 4°C for 30 min the labeling was uniformly seen on the whole cell (fig.1a). However, if the cells were further incubated at 37°C the labeling clustered, and after 20 mn, the fluorescence of the largest number of labeled cells concentrated at one cell pole as expected in a capping process (fig.1b). This result supports the idea that the sugar binding proteins detected in these cells are membrane proteins.

The presence of sugar binding proteins in the membrane of thymus and spleen cells is limited to some subpopulations only; this phenomenon is observed for each type of sugar tested, which suggest that these membrane proteins are not enzymes such as glycosyl transferases.

In conclusion, fluorescent glycosylated markers are shown to be suitable to visualize cell surface lectins and to characterize various spleen and thymus populations on the basis of the presence of specific lectins.

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