Detection of Sialic Acid on Cultured Cells by Binding of a Lectin From *Limax flavus*¹

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The surface of the glomerular visceral epithelial cells (GEC) is covered by an anionic coat, the main constituent of which has been shown to be *N*-acetyl neuraminic acid (sialic acid) (1,2). We were unable to detect sialic acid in monolayers of GEC by the thiobarbituric acid assay probably because of the minute amounts present. Miller et al. have previously reported that erythrocytes were agglutinated by a lectin from the slug *Limax flavus* (LFA) and that this could be inhibited by *N*-acetyl and glycolytic neuraminic acids (3,4). We have devised an assay that uses labeled LFA to explore relative changes in the total sialic acid content of GEC in vitro.

METHODS

The LFA Binding Assay

The GEC were maintained in culture with RPMI-1640 and 20% fetal bovine serum (5,6). The lectin (Calbiochem, La Jolla, CA) was labeled with ¹²⁵I by the chloramine-T method (7). GEC were grown to confluence in 24-well dishes (Corning, Corning, NY) and were washed gently in 0.02 M phosphate buffer made in 0.135 M sodium chloride (PBS). To each well, 0.2 mL of PBS containing 0.1% gelatin (PBS-G) as well as [¹²⁵I]LFA in 50 µL (approximately 25,000 cpm) were added. After incubation for 1 h at room temperature on a shaker platform, the cell layers were washed gently in PBS and solubilized in 1 mL/well of 0.1% sodium dodecyl sulfate (BioRad, Richmond, CA), and the radioactivity was estimated with a gamma counter (LKB-Bromma, Sweden). To study the effects of fixation, the lectin binding was measured in cells fixed in 95% ethanol. Because the fixed cells could not be solubilized completely in sodium dodecyl sulfate, the cell layers were scraped by cotton tips and the tips were counted.

Competitive Inhibition of LFA Binding to GEC by Test Substances

The specificity of the assay was evaluated by testing the effects of *N*-acetyl neuraminic acid (0.625 to 10 mg/mL; 2 × 10⁻³ to 32 × 10⁻³ M) and 100 mg/mL of each of the following sugars: glucose (0.56 M), galactose (0.56 M), mannose (0.56 M), fructose (0.56 M), *N*-acetyl glucosamine (0.45 M), α-methyl mannanside (0.3 M), and sucrose (0.29 M). Gelatin and BSA were employed in 10 mg/mL concentrations. The [¹²⁵I]LFA was preincubated with equal volumes of the above test substances for 1 h at room temperature. Confluent layers of GEC were incubated with 50 µL/well of the preincubated lectin and 200 µL/well of PBS-G for 1 h at room temperature. The LFA binding was measured as described above.

The effects of sialoglycoproteins on LFA binding were also tested. Confluent layers of GEC were incubated for 1 h or overnight at 4°C with [¹²⁵I]LFA in PBS-G in the presence of various concentrations of mucin (bovine submaxillary, type 1) (0.1 to 10 mg/mL; 0.25 × 10⁻⁶ M to 25 × 10⁻⁶ M), gelatin (0.1 to 10 mg/mL), and fetuin (0.125 to 2 mg/mL; 2.77 × 10⁻⁶ M to 4.32 × 10⁻⁵ M). After being rinsed in PBS, the LFA binding was measured as described above.

Neuraminidase Treatment

Confluent layers of GEC fixed in alcohol were incubated for 6 h at 37°C with 40 mIU/mL of neuraminidase (from *Vibrio cholerae* Sigma Chemical Co., St. Louis, MO) in 50 mM acetate–0.14 M NaCl (pH 5.5) buffer containing 10 mM calcium chloride and 1 mM phenylmethylsulfonyl fluoride. The monolayers were washed and [¹²⁵I]LFA binding was measured as described above.
RESULTS AND DISCUSSION

The GEC readily bound LFA, the fraction bound accounting for 10 to 15% of the label added. The interassay variability in the fraction of label bound was 11.5 ± 5.51% (mean ± SD of 21 experiments performed over 2 yr with seven separately labeled batches of [125I]LFA). The intra-assay coefficient of variability with respect to bound counts per million/total counts per million was approximately 6%. The assay could be performed with ease on unfixed cells for 1 h at room temperature or for 3 h at 4°C, thus permitting observations on sialic acid residues expressed on the apical cell surface. The fixation of cells in ethyl alcohol increased the lectin binding by a factor of 1.8 compared with that of unfixed cells. Alcohol fixation may permit access of the lectin to sialic acid residues located in deeper layers of the cell membrane.

The binding of the labeled lectin was inhibited by N-acetyl neuraminic acid in a dose-dependent manner (2 × 10⁻³ to 32 × 10⁻³ M) (Figure 1). In contrast, 10-fold higher concentrations of glucose, galactose, mannose, fructose, α-methyl mannoside, and N-acetyl glucosamine failed to demonstrate any inhibition. Gelatin and BSA also failed to inhibit lectin binding. The selective inhibition of LFA binding to the GEC monolayers by N-acetyl neuraminic acid and the failure to do so by 9- to 17.5-fold higher molar concentrations of sugars commonly found in glycoproteins suggested that LFA specifically bound to sialic acid residues on the cell surface of the GEC.

The specificity of the lectin binding was further tested with sialoglycoproteins. Whereas fetuin and mucin caused a dose-dependent inhibition in LFA binding to monolayers of GEC, gelatin, a nonsialated glycoprotein, had no effect. Significant inhibition of LFA binding (39%) by mucin could be seen at 0.1 mg/mL (0.25 × 10⁻⁶ M); maximum inhibition was seen at 1 mg/mL (2.5 × 10⁻⁶ M) (90%), with a plateau at higher concentrations. With the nonspecific binding data, the signal-to-noise ratio was calculated to be approximately 7. The lectin binding was more sensitive to inhibition by sialoglycoproteins than by soluble N-acetyl neuraminic acid, probably because of a more favorable conformational presentation of sialic acid residues by the former.

The effect of neuraminidase treatment on LFA binding was evaluated next. Detachment of GEC was observed at the optimal pH required for neuraminidase activity (pH 5.5). The assay was, therefore, performed on alcohol-fixed monolayers. Neuraminidase caused approximately 70 to 80% reduction in LFA binding: control (N=3), 1,820 ± 97 cpm/well (mean ± SE), versus neuraminidase, 354 ± 67 cpm/well (P<0.001). Twofold higher concentrations of the enzyme or prolongation of the incubation to 24 h did not result in a greater reduction in lectin binding. These data further confirmed the specificity of LFA binding to sialic acid. The inability of neuraminidase to completely inhibit lectin binding may be because of the incomplete removal of sialic acid residues by the enzyme.

The major sialoprotein of the GEC is podocalyxin, a 140-kd, sulfated molecule (8,9). Sialoglycoproteins may be important in the maintenance of the intricate pedicel structure of the GEC, as suggested by the association of the fusion of pedicels with the loss of sialic acid from the GEC in experimental nephrosis (10). Podocalyxin is a marker of the differentiation of GEC in the developing kidney (11). The availability of GEC in homogeneous cultures (5,6) and the development of a specific LFA assay that facilitates the detection of minute amounts of sialic acid permit studies on the cellular metabolism of sialoglycoproteins. Furthermore, this method should be applicable to sialic acid studies on any other cell line. This method is valuable as a screening assay for the detection of the overall changes in the sialic acid content of cells in vitro.

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