Carbohydrate induced modulation of cell membrane: II. Spin label study of fluidity changes in peripheral blood lymphocyte membrane

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Abstract This paper reports for the first time, that binding of various mono-, di-, and trisaccharides to membrane lectins reduces the rotational motion of membrane proteins and lipids indicating a decrease in membrane fluidity as studied by EPR spectroscopy using spin probes. Interaction of polysaccharides with lymphocyte resulted in an extensive decrease in membrane fluidity making the membrane almost rigid. The decrease in fluidity was dose-dependent, dependent on the multivalency of the ligand used, and was sensitive to presence of EDTA and sodium azide. Binding of two different carbohydrate ligands on their respective surface lectins has a synergistic effect on the decrease in membrane fluidity.

Key words: Lymphocyte membrane; Selectin; Fluidity; EPR

1. Introduction

Numerous reports have appeared delineating the role of selectin in lymphocyte homing [1–3], but the physiological changes in the lymphocytes after binding of the selectin ligands have never been studied. Some recent reports do suggest that the cytoplasmic domain of selectins may be involved in signal transduction [4–6]. The main aim of our work was to monitor biophysical changes in lymphocyte membrane subsequent to selectin–ligand interaction.

Earlier, we detected the presence of a sialic acid-binding lectin and a glucose-binding lectin on goat peripheral blood lymphocytes (GPBLs) [7]. We also studied the biophysical changes following lymphocyte–sialic acid interaction occurring at the lymphocyte membrane using EPR spectroscopy. Our preliminary studies indicated that binding of sialic acid or mucin to GPBLs markedly reduces the rotational mobility of membrane proteins as well as that of lipids [8]. The results obtained demanded a detailed investigation in this area as it was not clear whether this was a specific effect produced by interaction of lymphocytes with sialic acid or its derivatives only or was a generalized effect produced by the interaction of membrane lectins with their respective ligands. We have now monitored the effect of various mono-, di- and oligosaccharide exposures on intact lymphocyte membranes using EPR spectroscopy. The spin label probes used were 5 DS, 16 DS, MECP and TEMPO. The stearic acid spin labels 5 DS and 16 DS were used as probes for the hydrocarbon portion of the membrane bilayer. TEMPO was used as a probe for the aqueous compartments while MECP was used to probe the surface portion with reactive NH_2 group. EPR spectra obtained were analysed by computing the rotational correlation time (T_c). T_c is the measure of degree of immobilization of the spin label, hence a measure of the local viscocity. Our hypothesis is that the specific binding of carbonhydrates to membrane surface lectins (selectins) induces an energy-dependent stiffening of the lipid membrane.

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Abbreviations: 5-DS, Glc NAc, *N*-acetylglucosamine 5-doxyl-stearic acid; 16-DS, 16-doxyl stearic acid; MECP, 3-(2-maleimidoethyl carbomyl) proxyl; TEMPO, 2,2,6,6 tetramethyl-1 piperidine *N*-oxyl; PBS, 0.01 M phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.01 M $CaCl_2$; GPBL, goat peripheral blood lymphocyte.

2. Materials and methods

2.1. Reagents

Histopaque-1077, sialic acid, spin labels, i.e. 5-DS, 16-DS, TEMPO and MECP, and all the sugars were procured from Sigma Chemical Co., MO, USA. The glass capillaries were from TOP Syringe Mfg. Co., Bombay, India. All the other reagents were of analytical grade.

2.2. Method

GPBLs were isolated using Histopaque-1077, treated with various mono-, di- and oligosaccharides and spin labeled with either 5-DS, 16-DS, MECP and TEMPO as described earlier [7,8]. The carbohydrates and their complex forms used were glucose, *N*-acetylglucosamine, maltose, mannose, mannose-6-phosphate, galactose, maltotriose, fucoidan, hyaluronic acid, sialic acid and heparin.

EPR spectra were recorded on a Varian E-104 EPR spectrometer equipped with a TM_{110} cavity. Instrument settings employed were as follows: scan range 100 G, field set 3237 G, temperature 27°C, time constant 1 s, scan time 1 s, modulation amplitude 2 G for 16-DS and TEMPO and 1 G for 5-DS and MECP, modulation frequency 100 kHz, microwave power 5 mW, microwave frequency 9.01 GHz, receiver gain 2.5×10^4. The rotational correlation time T_c and order parameter S_3 were calculated as described earlier [8]. The experiments were repeated 4 to 6 times and data were subjected to statistical analysis using the STAT-P-GW-BASIC Rev 1.02 programme.

3. Results

The changes in $1/T_c$ values of different spin labels after interaction of lymphocytes with various carbohydrates are summarized in Table 1. It was found that binding of glucose to the membrane lectin results in a decrease in fluidity of the lymphocyte membrane as denoted by decrease in $1/T_c$ values of all the spin probes used. The EPR spectra showing the mobility, respectively, of MECP, 5-DS and 16-DS incorporated in GPBL treated with various sugars are represented in Figs. 1 and 2, respectively. A small, however significant, decrease is observed in the rotational dynamics of membrane proteins. More pronounced decrease is observed in the lipid domain. The $1/T_c$ value for 5-DS reporting the microenvironment near the surface region (6 Å from carboxyl group) [9] of the lipid bilayer decreased from 0.79 to 0.39. A maximum (fourfold) decrease was however reported by 16-DS probing the inner core (18 Å from the carboxylic group) [9] of the lipid bilayer where the $1/T_c$

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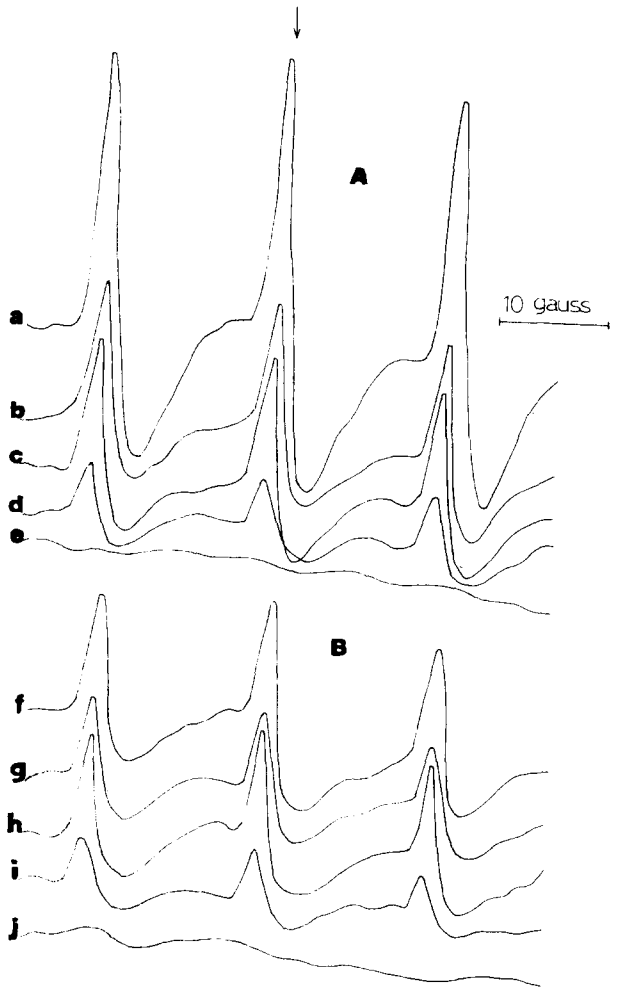

Fig. 1. (A) EPR spectra of GPBL labelled with MECP incubated in (a) PBS as control, (b) glucose, (c) *N*-acetyl D-glucosamine, (d) maltose, (e) maltotriose. (B) EPR spectra of GPBL labelled with 5-DS incubated in (f) PBS as control, (g) glucose, (h) *N*-acetyl D-glucosamine, (i) maltose, (j) maltotriose. Instrument settings were as described in section 2.

value changed from 1.19 to 0.29 (Table 1). Changes were not observed in the order parameter S_3. As 5-DS and 16-DS are stearic acid derivatives, the chance that they are in an aqueous environment is remote and any such label hanging in the buffer will be totally quenched due to the addition of paramagnetic Ni^{2+} ions. The dose-dependent decrease in fluidity in respect to the addition of glucose in increasing concentrations from 0.006 mM to 1.25 mM (final conc.) was also studied. On increasing the glucose concentration, there is a subsequent decrease in membrane fluidity with saturation being achieved at 0.125 mM glucose. A tenfold increase in glucose concentration produced no further decrease in membrane fluidity.

Binding of maltose, a disaccharide with repeating glucose units, produced a 7.6-fold decrease in fluidity as probed by 16-DS. A similar decrease in fluidity was also observed when lymphocytes were incubated with GlcNAc, mannose, mannose-6-phosphate and fucose (Table 1). With maltotriose, a trisaccharide with repeating glucose units, hyaluronic acid and heparin, there was total immobilization of the spin probes resulting in a flat spectra. Since GPBL were shown to express both sialic acid- and glucose-binding proteins [7] it was also speculated that interaction of sialic acid and glucose together with the lymphocytes might have a synergistic effect on the decrease of membrane fluidity. The results indicate that separately glucose and sialic acid both do decrease the membrane fluidity but when added together, the membrane fluidity decreases extensively to the extent of making the membrane totally rigid, with full immobilization of the spin probe, producing a flat spectra (Fig. 3).

The spin probe TEMPO used in this study is water-soluble and partitions in a heterogeneous environment in which both hydrocarbon and aqueous zones co-exist [10]. A large proportion of the probe in the membrane spends, on average, a longer time close to the membrane–water interface, thus reporting about the cytoplasmic viscosity; the probe on the extracellular interface being quenched by paramagnetic ion such as Ni^{2+} [11]. Since nitroxide has a greater hyperfine coupling in polar domains than in non-polar domains as well as a smaller G value in polar areas, the spectral line positions do not exactly align [12] (Fig. 2, $aN \neq aN'$). This also clearly indicates that TEMPO is localized in an aqueous environment, whereas 5-DS or 16-DS are localized in a lipid environment.

Surprising results were obtained when TEMPO was used as the label. With all the sugars used, which are producing de-

Table 1
Changes in fluidity (represented as $1/T_c \times 10^{10}$ s^{-1}) in different regions of GPBL after treatment with various sugars*

Spin probe used	PBS	Glu	PBS	GluNAc	PBS	Mal	PBS	Man	PBS	$Man6PO_4$	PBS	Fuc	PBS	Gal
MECP	1.77	1.17 $P<0.01$	1.78	1.29 $P<0.01$	1.78	1.29 $P<0.02$	1.71	1.42 NS	1.63	1.27 $P<0.02$	1.95	1.31 $P<0.001$	1.72	1.72 NS
5-DS	0.79	0.39 $P<0.001$	0.74	0.38 $P<0.001$	0.78	0.49 $P<0.001$	0.61	0.38 $P<0.01$	0.57	0.44 $P<0.02$	0.74	0.37 $P<0.001$	0.58	0.58 NS
16-DS	1.19	0.29 $P<0.001$	1.19	0.31 $P<0.001$	1.14	0.15 $P<0.001$	1.00	0.156 $P<0.001$	1.21	0.175 $P<0.01$	1.28	0.20 $P<0.001$	1.84	1.86 NS
TEMPO	1.38	0.33 $P<0.001$	1.63	0.33 $P<0.001$	1.96	0.33 $P<0.001$	1.10	0.32 $P<0.001$	0.92	0.73 $P<0.05$	1.22	0.33 $P<0.001$	0.97	0.97 NS

*All experiments were repeated 6 times independently using PBS as control each time. Incubation with maltotriose, heparin and hyaluronic acid produced totally flat spectra and thus $1/T_c$ values could not be calculated for them. (Glu: glucose, GluNAc: *N*-acetylglucosamine, Mal: maltose, Man: mannose, $Man6PO_4$: mannose 6-phosphate, Fuc: fucoidan, Gal: galactose, PBS: phosphate buffered saline, NS: not significant.)

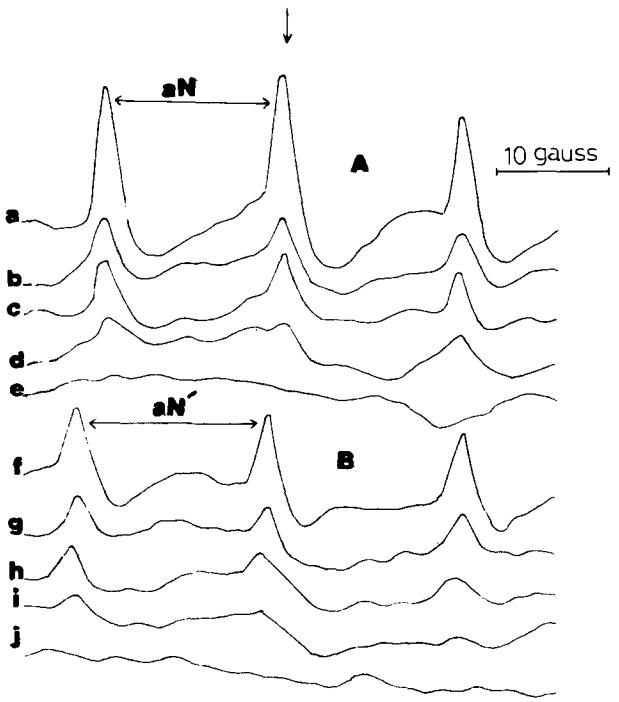

Fig. 2. (A) EPR spectra of GPBL labelled with 16-DS incubated with (a) PBS as control, (b) glucose, (c) *N*-acetyl D-glucosamine, (d) maltose, (e) maltotriose. (B) EPR spectra of GPBL labelled with TEMPO incubated with (f) PBS as control, (g) glucose, (h) *N*-acetyl D-glucosamine, (i) maltose, (j) maltotriose.

crease in GPBL membrane fluidity, a decrease in $1/T_c$ values for TEMPO was also seen, indicating an increase in cytoplasmic viscosity (Fig. 2B). On incubation with polysaccharides with multiple binding sites such as maltotriose (Fig. 2B) or heparin or hyaluronic acid, complete immobilization of TEMPO was seen as indicated by a flat spectra. TEMPO is known to be catabolized by certain cells [13]. To assure that the broadening of the hyperfine splittings was due to catabolic breakdown or due to immobilization, we sonicated the TEMPO labeled cells incubated with heparin showing complete immobilization of TEMPO. Sonication resulted in reappearance of TEMPO signals (Fig. 4) indicating that the probe was not catabolized but immobilized.

As the glucose binding lectin was Ca^{2+}-dependent we studied if the presence of EDTA inhibited the decrease in membrane fluidity. Before incubation with glucose, the cells were preincubated with 1 mM EDTA in PBS for 10 min. Cells treated with glucose in the presence of EDTA gave a $1/T_c$ value of 0.40 for 16-DS, whereas those treated with glucose in the absence of EDTA gave a $1/T_c$ value of 0.13 exhibiting a 3-fold decrease in fluidity (Fig. 4). This clearly showed that EDTA inhibited the binding of glucose to surface lectins as well as inhibiting the decrease in membrane fluidity.

We also tested if it was necessary for the cells to be metabolically active and live to produce changes in membrane fluidity. The cells were preincubated for 30 min in PBS containing 20 mM sodium azide. Cells were then washed thrice, resuspended in PBS and incubated with glucose as in the normal protocol. Sodium azide-treated cells did not exhibit the decrease in membrane fluidity as exhibited by untreated cells used as controls (Fig. 4). The $1/T_c$ value of MECP was found to be 0.85 for cells incubated in PBS alone, 0.74 for glucose-treated cells preincubated in 20 mM sodium azide and 0.20 for glucose-treated cells not incubated in sodium azide.

Our earlier binding studies indicated that the GPBL membrane does not contain any galactose binding protein [7]. Accordingly, we assumed that incubation of GPBL with galactose should not produce any changes in lymphocyte fluidity. Our results are in concurrence with this assumption and no change in lymphocyte membrane fluidity or in cytoplasmic fluidity was observed on incubation with galactose (Table 1).

4. Discussion

Our results indicate that binding of carbohydrates or their derivatives to GPBL surface lectin results in a decrease in membrane fluidity as well as an increase in cytoplasmic viscosity. The decrease in fluidity of the membrane or cytoplasm is enhanced by the increase in multivalency of the ligand possibly due to cross-linking of surface lectins. Thus, in comparison with glucose, maltose produces more broadening of lines and incubation with maltotriose, heparin or hyaluronic acid completely flattens the spectra indicating a total immobilization of the probes. This also indicates that the cells surface lectins are

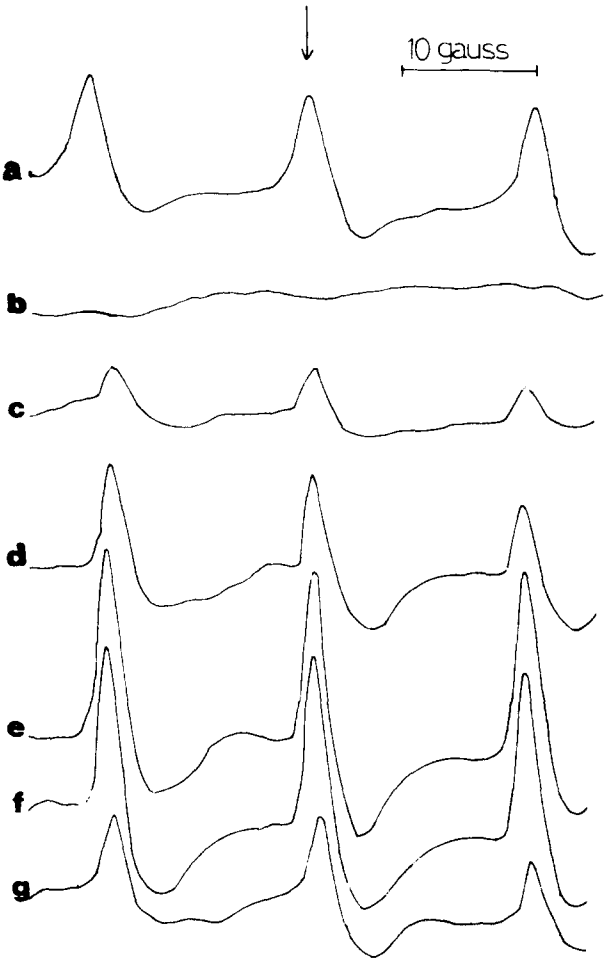

Fig. 3. EPR spectra of GPBL labelled with 16-DS incubated with (a) PBS as control $1/T_c$ value 1.20, (b) sialic acid $1/T_c$ value 0.14, (c) glucose $1/T_c$ value 0.30, (d) sialic acid and glucose $1/T_c$ value could not be calculated as total broadening of the lines has occurred.

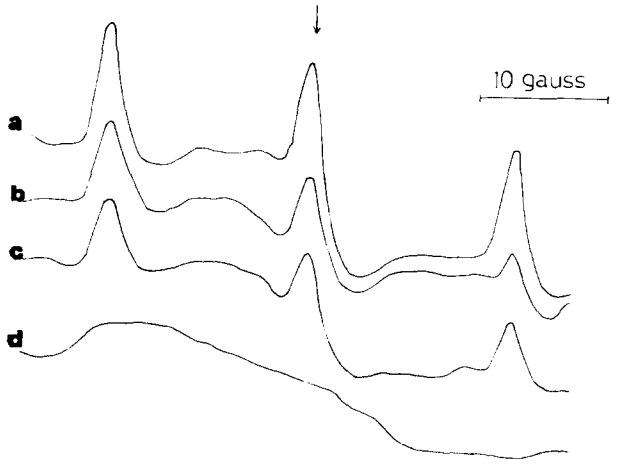

Fig. 4. EPR spectra of GPBL labelled with TEMPO. (a) incubated with heparin followed by sonication, (b) incubated with heparin, not sonicated, (c) GPBL labelled with 16-DS incubated with glucose, (d) incubated with glucose in the presence of 10 mM EDTA, (e) GPBL labelled with MECP incubated with PBS, (f) GPBL preincubated in 20 mM sodium azide followed by incubation in glucose, (g) incubation in glucose alone.

present close to each other on lymphocyte surface and are free to move in the bilayer enabling them to be cross-linked by a multivalent ligand. Binding of ligands (e.g. sialic acid or glucose) on two different surface lectins also results in a massive decrease in fluidity producing a flat spectrum (Fig. 3). Based on these results at least two speculations could be made. Firstly, the ligand binding changes the mutual interactions between proteins and lipids along the full length of the hydrocarbon chains resulting in a decreased bilayer fluidity, and secondly, the ligand binding transmits a signal to the cytoplasm producing some physiological changes. As no significant changes in S_3 or order parameter was found, we assume that lipids exist in specific lipid domains [14] and the ligand binding does not necessarily lead all the lipid chains to assemble themselves in a more ordered (rigid) fashion with a concurrent decrease of membrane fluidity.

The most surprising feature of our results is the complete immobilization of the aqueous probe TEMPO. It is postulated that a large portion of the cytoplasm is ordered by the interior membrane surface, but the mechanism of this ordering is not yet known. Although very rare, immobilization of TEMPO has been reported earlier in the case of rabbit sarcoplasmic reticular vesicles [11].

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