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# INTERACTION OF NEUTRAL POLYSACCHARIDES WITH HUMAN ERYTHROCYTE MEMBRANE: INVOLVEMENT OF PHOSPHOLIPID BILAYER

By MAURIZIO MINETTI, ANGELA TEICHNER and PATRIZIA ADUCCI
Laboratorio di Biologia Cellulare ed Immunologia
Istituto Superiore di Sanità
Viale Regina Elena 299
Roma-ITALY

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SUMMARY. The erythrocyte aggregating activity of different neutral polysaccharides depends on the polymer - membrane affinity and its molecular length. Extended proteolytic and neuraminidase treatments of red blood cell membrane did not reduce aggregation induced by polysaccharides. Fluorescent dextran studies showed that polymer adsorption on red blood cells was measurable only for a dextran molecular weight above 40,000 as for cell aggregation. Identical dextran adsorption behaviour was observed for all liposomes composed of various purified phospholipids, suggesting the involvement of the phospholipid bilayer in dextran-membrane interaction. All aggregations induced by neutral polysaccharides were inhibited by D-and L-tryptophan. Compounds very similar to tryptophan were less active or completely inactive inhibitors. Equilibrium dialysis, gel filtration and tryptophan fluorescence studies clearly showed that there was no complex formation between tryptophan and neutral polysaccharides. Therefore tryptophan does not result an haptenic inhibitor, but reduces the adsorption of polysaccharides on cell membrane.

The biological role of cell surface polysaccharides is an aspect of membrane biology which is still not fully understood. Speculations have been made as to their role in the regulation of cell proliferation and cell-cell communication(1). A possible approach to an investigation into the arrangement of surface polysaccharides on the membrane and polysaccharide involvement in biological processes is the study of the interaction between cell surface and model polysaccharides.

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It is known,in fact,that neutral polysaccharides can induce some biological processes such as lymphocyte mitogenesis (2), cell aggregation (3-5),cell fusion (6),but little has been said about the membrane constituents involved in the interaction with exogenous polysaccharides. This paper studies the aggregating activity of different neutral polysaccharides on the human erythrocyte . Evidence reported suggests that the phospholipid bilayer is responsible for the interaction between red blood cells and neutral polysaccharides. The red blood cell capacity to adsorb neutral polysaccharides can be reduced in the presence of D- or L-tryptophan.Preliminary experiments to clarify the mechanism of tryptophan inhibition activity have been carried out.

## MATERIALS AND METHODS.

Chemicals. The reagents used were:dextrans T fractions(molecular weights from 10,400 to 2,000,000),ficoll 400(molecular weight of about 400,000) and fluoresceinylthiocarbomoyl-dextrans(FITC-dextrans)with molecular weight ranging from 2,820 to 154,000 (Pharmacia,Uppsala,Sweden); papain,D- and L-tryptophan,5-hydroxy-L-tryptophan,N-α-acetyl-DL-tryptophan,DL-phenylalanine,L-tyrosine (Merck,Darmstadt,Germany) ; 5-methyl-DL-tryptophan,glycyl-L-tryptophan and L-leucyl-L-tryptophan(K&K Plainvew,N.Y.,USA) ; DL-5-benzyloxytryptophan(Sigma,St.Louis, USA) ; L-β,γ-distearoyl-α-lecithin,L-β,γ-distearoyl-α-cephalin and trypsin (Fluka,Buchs,Switzerland) ; pronase(Strept. griseus)and neuraminidase (Vibrio cholerae) (Calbiochem,Calif. USA) ; bromelin(Pineapple)(Behringwerk AG,Marburg-Lann,Germany) ;Bio-Gel P-2 (Bio-Rad,Richmond,Calif.,USA) ; sphingomyelin (bovine) and phosphatidyl serine (bovine) (Supelco,Inc.Pennsylvania,USA).

Cell aggregation and inhibition. Fresh human erythrocytes were washed three times by centrifugation in saline,the leucocytes being removed by aspiration after each centrifugation. Agglutination and inhibition tests were performed,as previously described (5),by the microtiter system(Dynatech Lab. Ltd., Sussex,U.K.). Soluble inhibitors and D- and L-tryptophan were tested at a concentration of 70 mM. Poorly soluble inhibitors were tested at saturation.

Enzymatic treatments. Trypsin treatment of erythrocytes was as previously described (5) with 0.05% trypsin at 37°C for 2hours. Pronase treatment was performed, according to the method of Bender et al.(7), at a concentration of 0.01% at 37°C for 2 hours, either on freshly prepared erythrocytes or on trypsinized red blood cells. Neuraminidase was tested on erythrocytes at a concentration of 50U/ml at 37°C for 60 min. according to Schnebli et al.(8). Bromelin and papain digestions were carried out at a concentration of 1mg/ml at 37°C for 30 min. In all enzymatic treatments the cell concentration was $2x10^8$ cells/ml.

Phospholipid liposomes. The phospholipids used in this study were chromatografically pure. In order to obtain multilamellar dispersion each phospholipid was suspended in phosphate buffered saline pH 7.4 at a concentration of 5 mg/ml, heated and shaken above the crystal-to-liquid crystal transition temperature for a few minutes and finally allowed to stand overnight at room temperature. It is known that phosphatidylethanolamine formed incompletely sealed particles (9). To test phosphatidylethanolamine we used an admixture of phosphatidylcholine with phosphatidylethanolamine at a ratio of 1:1 that resulted in the usual closed structures (9).

Adsorption of fluorescent dextrans on erythrocytes. The binding of fluorescent dextrans was performed by adding 5mg of FITC-dextrans to 1ml of erythrocytes ($1x10^8$ cells/ml) suspended in Ringer-Locke buffer (10) or in 1 ml of the same buffer containing 5 mg of phospholipid-liposomes . The distribution of fluorescence was observed using a Leitz Orthoplan fluorescence photomicroscope. The erythrocytes and liposomes were equilibrated in Ringer-Locke buffer containing 70 mM tryptophan prior to addition of FITC-dextran 150 in tryptophan inhibition experiments.

RESULTS AND DISCUSSION.

Aggregating activity of neutral polysaccharides. Polysaccharide-induced erythrocyte aggregation was determined as the minimal concentration of polymer that induces detectable cell clumping. At low polymer concentration, in fact, the cell aggregation depends mainly on the adsorption force of the polymer on the cell membrane and is quite independent of the electrostatic repulsion force (11). In agreement with the data reported (3,4,11) dextran causes erythrocyte aggregation only above a molecular weight of 39,500 and the minimal aggregating

Table I. Aggregating activity of different polysaccharides on human erythrocytes ($1x10^8$ Cells/ml)

| Polysaccharide | Mw[b] | Minimal aggregating concentration (mg/ml)[a] | |
|---|---|---|---|
| | | Buffered Saline (pH 7.4) | Buffered Saline + 70mM D- or L-tryptophan |
| Dextran T-10 | 10,400 | inactive | - |
| " T-40 | 39,500 | 50[c] | - |
| " T-70 | 70,000 | 12.5 | 50 |
| " T-110 | 106,000 | 6.3 | 25.2 |
| " T-150 | 154,000 | 3.2 | 12.8 |
| " T-250 | 231,000 | 1.5 | 12.0 |
| " T-500 | 496,000 | 1.5 | 12.0 |
| " T-2000 | 2,000,000 | 1.3 | 10.4 |
| Ficoll 400 | 400,000 | 3.1 | 9.3 |
| Arabinoxylan | 50,000 | 0.6 | 9.6 |

[a] Minimal aggregating concentration is the lowest concentration of polysaccharide showing detectable cell clumping.

[b] Dextran and ficoll molecular weights are manufacturer's data.

[c] Very weak aggregation.

concentration decreases with increasing dextran molecular weight (Table I). However the affinity of the polymer for red blood cell membrane can play a more significant role than molecular length. In fact Wheat arabinoxylan with a molecular weight of only 50,000 (5) showed an aggregating activity as great as that of dextran with a molecular weight of 2,000,000 (Table I). In addition, as shown in Table I, the aggregating activity of ficoll (highly branched synthetic polysaccharide) was less than that shown by dextran (low branched polysaccharide[4]) with an analogous molecular weight. This suggests that the branching <u>per se</u> is not the most important prerequisite for aggregating activity.

TABLE II. Effect of proteolytic and neuroaminidase treatments of human erythrocyte membrane on polysaccharide-induced cell aggregation.

| Treatment | Minimal aggregating concentration (mg/ml) | |
|---|---|---|
| | Dextran T-2000 | Wheat Arabinoxylan |
| None | 1.3 | 0.6 |
| Pronase | 0.6 | 0.3 |
| Trypsin | 0.6 | 0.6 |
| Trypsin+Pronase | 0.6 | 0.3 |
| Papain | 0.6 | 0.6 |
| Neuroaminidase | 0.3 | 0.3 |
| Bromelin | 0.3 | 0.6 |

Similar results were obtained with the other polysaccharides listed in Table I.

Membrane constituents involved in dextran binding. In order to identify the membrane constituents involved in polysaccharide binding we considered the proteic membrane receptors and the phospholipid bilayer. The hypotetical presence on the erythrocyte membrane of a proteic or glycoproteic receptor specific to polysaccharides seems to be excluded by the observation that extended treatments of erythrocyte membrane with proteolytic enzymes did not decrease polysaccharide-induced aggregations (TableII). It is known, in fact, that exhaustive treatment of erythrocytes with trypsin and pronase removes the only two proteins exposed on the external surface (13,14). The neuraminidase treatment shows that sialic acid residues are not involved in neutral polysaccharide-membrane binding. The

slight increase in the aggregating activity of polysaccharides (TableII) could be due to the displacement of the negative charge of the red cell surface as a consequence of the enzymatic treatments (8). The involvement of the lipid bilayer in membrane-polysaccharide interaction was investigated by adsorption studies with several fluorescent dextrans. As reported by other authors (10,15),the dextran adsorption on the erythrocyte membrane is very weak and affected by cell washing. Fig.1 shows the faint fluorescence obtained with FITC-dextran 150 adsorbed either on the erythrocyte (Fig.1a)or on the lecithin-liposome (Fig.1b). Table III summarizes the adsorption evaluation on both erythrocytes and liposomes composed of various phospholipids. The used widely occurring lipids are the major constituents of the red blood cell membrane (14). The minimal molecular weight of dextran necessary to induce stable membrane adsorption (Table III)was practically the same as that reported in Table I for erythrocyte aggregation. Table III shows also a good correlation between erythrocytes and all the phospholipid-liposome tested. The last two points imply,therefore, that the interaction between polysaccharides and erythrocytes can occur with phospholipidic membrane components at the level of the lipid-water interface. Concerning the proposed involvement of the phospholipid bilayer in polysaccharide-membrane interaction it is interesting to note that, at least in cell fusion a direct participation of the phospholipid bilayer seems to be established (16-18).

Inhibition of polysaccharide-induced cell aggregation. All the aggregations induced by the neutral polysaccharides tested were inhibited by the same compound:D- or L-tryptophan (Table I). The usual haptenic inhibitors of plant lectins (19,20) and the constituent carbohydrates of polysaccharides (D-glucose for dextran,sucrose for ficoll,D-xylose and L-arabinose for wheat arabinoxylan (5)) were ineffective as inhibitors. Moreover, tryptophan seems to be a specific inhibitor and in fact com-

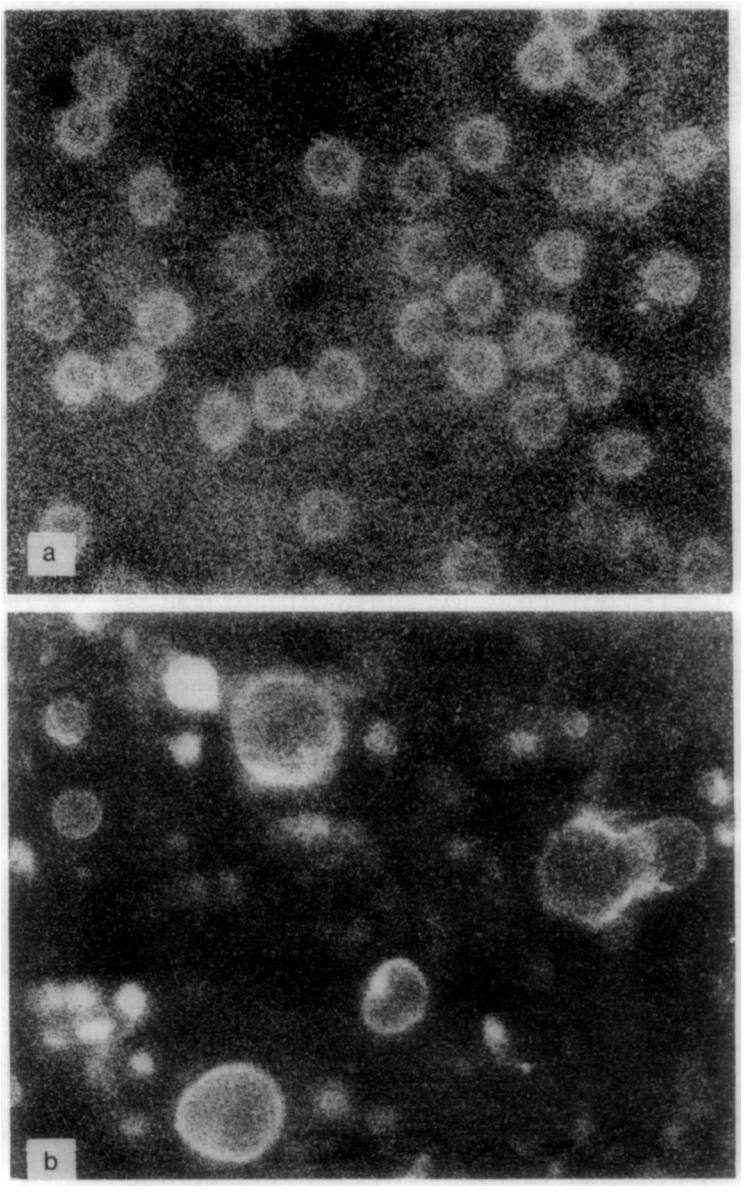


Fig.1 Fluorescence photomicrographs of FITC-dextran150 adsorbed on erythrocytes and Lecithin-liposomes.

Human erythrocytes($1x10^8$cells/ml) and Lecithin-liposomes were incubated for 30 min. with 5mg/ml of FITC-dextran150. After two washes with cold Ringer-Locke solution, the preparations were viewed under the microscope.
a) Human erythrocytes(1000X)
b)Lecithin-liposomes(700X)

Table III. Binding of FITC-dextrans on erythrocytes and phospholipid-liposomes

| Fluorescent dextrans | Mw[b] | Fluorescence (a.u.)[a] | |
|---|---|---|---|
| | | Erythrocytes | Liposomes[c] |
| FITC-dextran 3 | 2,820 | - | - |
| " 20 | 19,000 | - | - |
| " 40 | 39,000 | ± | ± |
| " 70 | 68,500 | + | + |
| " 150 | 154,000 | ++ | ++ |

[a](a.u.)=arbitrary units( - no fluorescence; ± hardly detectable fluorescence; + detectable fluorescence; ++ evident fluorescence).

[b]Molecular weights are manufacturer's data.

[c]Liposomes were composed of the following phospholipids: phosphatidylcholine; sphingomyelin;phosphatidylserine;and phosphatidylethanolamine-phosphatidylcholine(1:1). All phospholipids tested showed the same fluorescence pattern.

pounds such as 5-hydroxy-L-tryptophan and 5-methyl-DL-tryptophan were 4 times less active than D-or L-tryptophan;DL-5-benzyloxy-tryptophan and all the compounds substituted on the aminoacidic group such as N-α-acetyl-DL-tryptophan,glycyl-L-tryptophan and L-leucyl-L-tryptophan were completely ineffective as inhibitors. The aminoacids commonly present in proteins including aminoacids with an aromatic ring,like phenylalanine and tyrosine were also inactive. A possible tryptophan inhibition mechanism could be a complex formation with neutral polysaccharides,as reported for other agglutinating substances (19,20).In order to investigate the existence of such a complex,we studied the behaviour of tryptophan and polysaccharides in Bio-Gel P-2 gel filtration, in equilibrium dialysis and tryptophan fluorescence studies. All the data clearly excluded a complex formation in aqueous solution. The inhibiting activity of tryptophan was detectable also in adsorption studies on red blood cells with FITC-dextrans. Erythrocytes preincubated with 70mM D- or L-tryptophan exhibited no detectable fluorescence after binding with 5 mg/ml of FITC-dextran 150. The activity of tryptophan on cell aggregation thus seems an inhibition of the polymer adsorption on the cell

membrane.In spite of the good correlation between liposomes and erythrocytes in adsorption studies,phospholipid-membranes were not useful models for studying the inhibition of dextran-membrane interaction.In all the liposome concentrations tested (from 5mg to 0.5mg/ml)fluorescence intensity of adsorbed FITC-dextran 150 on liposomea was not reverted by tryptophan. Thus, a likely explanation of the inhibition mechanism is that the interaction of tryptophan with red blood cells may induce in the cell membrane changes that modify polymer adsorption.

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