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L-Fucose residues on cellulose-based dialysis membranes: quantification of membrane-associated L-fucose and analysis of specific lectin binding

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Contact of mononuclear human leukocytes with cellulose dialysis membranes may result in complement-independent cell activation, i.e. enhanced synthesis of cytokines, prostaglandins and an increase in β2-microglobulin synthesis. Cellular contact activation is specifically inhibited by the monosaccharide L-fucose suggesting that dialysis membrane associated L-fucose residues are involved in leukocyte activation. In this study we have detected and quantitated L-fucose on commercially-available cellulose dialysis membranes using two approaches. A sensitive enzymatic fluorescence assay detected L-fucose after acid hydrolysis of flat sheet membranes. Values ranged from 79.3 ± 3.6 to 90.2 ± 5.0 pmol cm^{-2} for Hemophan® or Cuprophan® respectively. Enzymatic cleavage of terminal α-L-fucopyranoses with α-L-fucosidase yielded 7.7 ± 3.3 pmol L-fucose per cm^2 for Cuprophan. Enzymatic hydrolysis of the synthetic polymer membranes AN-69 and PC-PE did not yield detectable amounts of L-fucose. In a second approach, binding of the fucose specific lectins of *Lotus tetragonolobus* and *Ulex europaeus (UEAI)* demonstrated the presence of biologically accessible L-fucose on the surface of cellulose membranes. Specific binding was observed with Cuprophan®, and up to 2.6 ± 0.3 pmol L-fucose per cm^2 was calculated to be present from Langmuir-type adsorption isotherms. The data presented are in line with the hypothesis that surface-associated L-fucose residues on cellulose dialysis membranes participate in leukocyte contact activation.

Keywords: biocompatibility, haemodialysis, dialysis membranes, L-fucose

Introduction

The activation of humoral and cellular blood components represents a major complication encountered during haemodialysis therapy of uraemic patients. The intensity and specificity of the interaction between the blood components and the dialysis membrane surface is closely associated with the type of membrane polymer selected [1]. In 1977, Craddock introduced the concept of *biocompatibility* for haemodialysis membranes, after he showed activation of the complement cascade during dialysis with the cellulose-based Cuprophan® (CU) membrane [2]. Complement activation has been incriminated in many clinical complications, but there are other mechanisms which may contribute to bioincompatibility, especially with cellulose-based membranes. Other potential reactions include protein adsorption, thrombogenic processes, activation of cells and humoral effector systems, together with the enhanced synthesis of β2-microglobulin. These may be stimulated either by inflammatory stimuli or by direct contact of blood cells with certain membranes (Fig 1) [3–5]. The latter may contribute to the occurrence of dialysis-related β2-microglobulin amyloidosis in long-term dialysed patients [5, 6]. We recently showed complement-independent blood cell activation after contact with CU, which resulted in the release of

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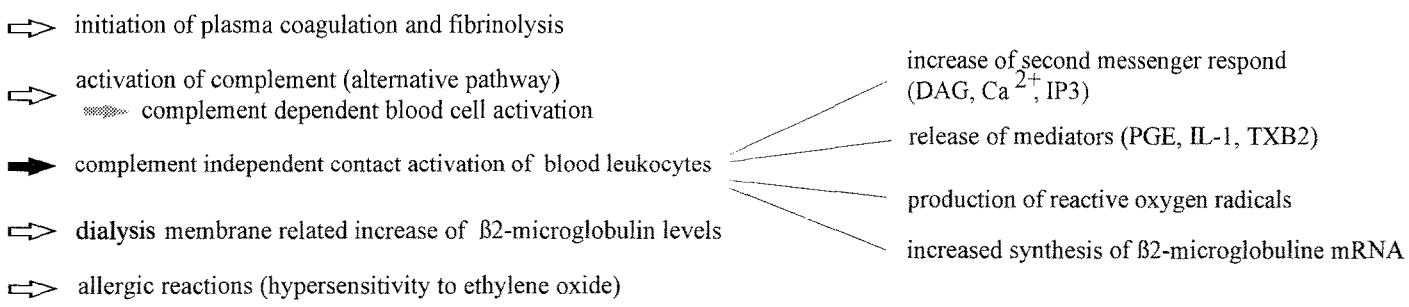

Figure 1. Activation of humoral and cellular blood components with dialysis membranes.

prostaglandins and interleukin-1, in the generation of oxygen radicals, and in an enhanced synthesis of β2-microglobulin [7, 8]. In parallel, we observed an increased turnover of cell membrane second messenger molecules, such as diacylglycerol and inositol phosphate as well as an increase in cytosolic calcium, after direct contact of human blood leukocytes with the membrane [9]. L-Fucose, but not D-fucose or other monosaccharides, inhibited cell activation [8, 9]. It was postulated therefore, that the dialysis membrane associated L-fucose residues may participate in cell activation.

In this study, the presence of membrane-bound L-fucose was directly assessed on CU and the DEAE-modified cellulose membrane, Hemophan® (HE). Recent efforts to determine fucose in membranes by GC/MS-analysis have been unsuccessful [9]. Based on an enzymatic cycling technique [10, 11] we are now able to detect and quantitate L-fucose in picomolar concentrations.

The presence of fucose on membranes was also assessed by the binding of fucose specific lectins of *Lotus tetragonolobus* and *Ulex europaeus* (UEAI). Two new synthetic polymer membranes were also included in our study for control purposes: these were the AN-69 membrane based on polyacrylonitrile, and the microdomaine structured polycarbonate-polyether (PC-PE, Gambrane®) membrane.

Materials and methods

Preparation of the dialysis membranes

Four different flat sheet membranes from commercially-available plate dialysers were used (Table 1). Circular pieces of membrane, 6 mm diameter, were punched out of the sheets and equilibrated with the appropriate buffer before use. Ten pieces were taken for the determination of bound fucose residues with the enzymatic fluorometric assay. For the lectin binding study, 16-well devices (7 mm inner diameter) were used; the membrane sheets were glued to one side of the polystryole module rings. Membranes were always washed and equilibrated with phosphate buffer containing 0.5% bovine serum albumin (PBS/BSA).

Measurement of L-fucose

The following reagents were used for the enzyme-fluorescence assay: L-Fucose dehydrogenase (lyophilized from porcine liver, approximately 0.67 U per mg protein) from Sigma; L-fucose, L-lactate dehydrogenase (from rabbit muscle, 476 U mg^{-1}), Adenosine-5'-diphosphate disodium salt, β-nicotinamide adenine dinucleotide, monohydrate, β-nicotinamide adenine dinucleotide reduced disodium salt trihydrate, 2-oxoglutaric acid, monosodium salt and L-lactic acid, sodium salt from Fluka, Neu Ulm; L-glutamate dehydrogenase (bovine liver, approximately 133 U mg^{-1}) from Calbiochem, α-L-fucosidase (EC 3.2.1.51, from beef kidney, approximately 2 U mg^{-1}) from Boehringer Mannheim. Unless stated all reagents were of highest possible grade.

Hydrolysis of membrane-associated L-fucose was performed as follows. Circular pieces of flat sheet CU, HE, AN-69 and PC-PE membranes were incubated with 0.25 ml of 0.1 M HCL for 1 h at 95 °C in tightly sealed glass vials. These conditions ensured optimal removal of the fucose and minimized the hydrolysis of the cellulose skeleton, which requires much harsher conditions in

Table 1. Dialysis membranes.

Polymer	*Type*	*Manufacturer*
Regenerated cellulose	Cuprophan	Akzo Co., Wuppertal, Germany
DEAE-modified cellulose	Hemophan	Akzo Co., Wuppertal, Germany
Polycarbonate-Polyether (PC-PE)	Gambrane	Gambro Co., Lund Sweden
Polyacrylonitrile	AN-69	Hospal Co., Meyzieu, France

terms of time, temperature and acid concentration. The membrane supernatants were cooled, neutralized with 1 M NaOH, and 0.2 ml of the neutralized solutions were pipetted into Eppendorf test tubes and stored at 4 °C until used.

Fucose was also removed from membranes by incubating the membranes in Eppendorf tubes overnight in 0.275 ml 0.1 M citrate/NaOH buffer, pH 5.5 and 50 mU of α-L-fucosidase (Boehringer, Mannheim). After hydrolysis, membranes were removed from the tubes to determine the residues that were not accessible to fucosidase. They were extensively washed with buffer containing 1% Tween, followed by vigorous washing with deionized water, dried at room temperature, and incubated with dilute HCl as described above. The supernatants from enzymatic hydrolysis was deproteinized by standing in a boiling water bath for 5 min, followed by 10 min freezing at −80 °C. Samples were quickly thawed out and centrifuged in an Eppendorf centrifuge for 2 min, and 0.2 ml of the supernatants were transferred into new Eppendorf tubes until needed.

The fluorescence assay relies on a cycling reaction first introduced by Lowry *et al.* [10] which was later modified by Morris [11]. We further modified the assay by adjusting the fucose dehydrogenase activity to 170 $mU\,ml^{-1}$, and eliminating the dilution with deionised water after the oxidation step to increase the sensitivity of the assay. NAD^+-fluorescence was generated after the addition of 6 M NaOH, and incubation at 60 °C for 10 min. Four ml of deionized water was finally added and the fluorescence was determined on a Perkin Elmer MPF 44 fluorescence spectrophotometer at 365 nm excitation wavelength and analysed at 457 nm.

L-fucose was quantitated with reference to freshly-prepared standards.

Lectin binding assay

Lectins from *Phaseolus vulgaris* (PHA), *Lotus tetragonolobus* (LTA), *Ulex europaeus* (UEA I) and their horseradish peroxidase (HRP) conjugates were from Sigma. Unless otherwise stated, all other materials were as described above.

The peroxidase-conjugated lectins were diluted to the appropriate concentrations with PBS containing 0.1% Synperionic PE/F 68 (Boehringer Mannheim, Germany) and pipetted into the 16-well plates with the membranes (CU, HE, AN-69 or PC-PE). The plates were incubated with gently shaking for 3 h at 37 °C in a moist-chamber. After this treatment the plates were washed carefully five times with PBS/Synperionic to remove unbound lectin. Subsequently, 0.1 ml 0.1 M citrate-phosphate buffer, pH 5, and 0.15 ml orthophenylene diamine ((PD) Dako Co., Hamburg, Germany) were pipetted into each well. After 5 min, the reaction was terminated by adding 0.05 ml 0.2 N H_2SO_4. Absorbance was measured at 429 nm wavelength against the reference wavelength of 620 nm using a microwell plate reader (EAR 340 AT from Lab Instruments, Austria).

A standard plot was prepared with respect to different concentrations of lectin. Standards diluted in citrate-phosphate buffer were pipetted into 96-well plates (0.1 ml per well) and the peroxidase reaction was carried out as described above.

Competitive-binding assay

Membranes were washed twice with the PBS/BSA buffer and incubated with different concentrations of UEA-I, LTA and PHA for 30 min at 37 °C. HRP-labelled UEA-I was added to the assay (100 μl per well) and competitive binding occurred during incubation at 37 °C for 3 h. Plates were extensively washed and peroxidase activity was assayed as previously described.

Avidin-gold labelled binding assay

Twenty-four well plates containing CU membrane were washed with sterile saline for 45 min at 37 °C and incubated with biotinylated LTA and PHA lectin (E-Y Laboratories, San Mateo, CA; purchased from Medac Co., Hamburg) at a final concentration of 10 $\mu g\,ml^{-1}$ in PBS, pH 7.4, for 90 min at 37 °C. After washing with PBS, non-specific binding sites were blocked by incubation with PBS containing BSA (5 $mg\,ml^{-1}$) for 30 min at 37 °C. Following further washing with PBS-BSA, the surfaces were incubated with avidin-gold complexes (20 nm, Sigma Chemical Co.) in PBS-BSA for 60 min at 37 °C, and then washed with PBS-BSA.

The following steps were performed before scanning electron microscopy: washing in distilled water, dehydration in ethanol, freeze drying and splutter-coating of a thin (approximately 5 nm) carbon/gold layer. The micrographs were made using secondary and back-scattered electron detection (BSE).

Results

Measurement of L-fucose associated with dialysis membranes

Hydrolysis of CU membranes released the maximum amount of free fucose after 1 h incubation under acid conditions at 95 °C (Fig. 2).

Results for the amounts of L-fucose released by acid hydrolysis of different membranes are summarized in Table 2. All samples were analysed in parallel with standards (Fig. 2) and blanks. For control purposes, membranes were also incubated with deionized water at 95 °C to evaluate the effect of the acid hydrolysis. These specimens gave fucose levels less than 5% of those obtained for the acid hydrolysed samples.

Differences between the CU and HE membranes (90.2

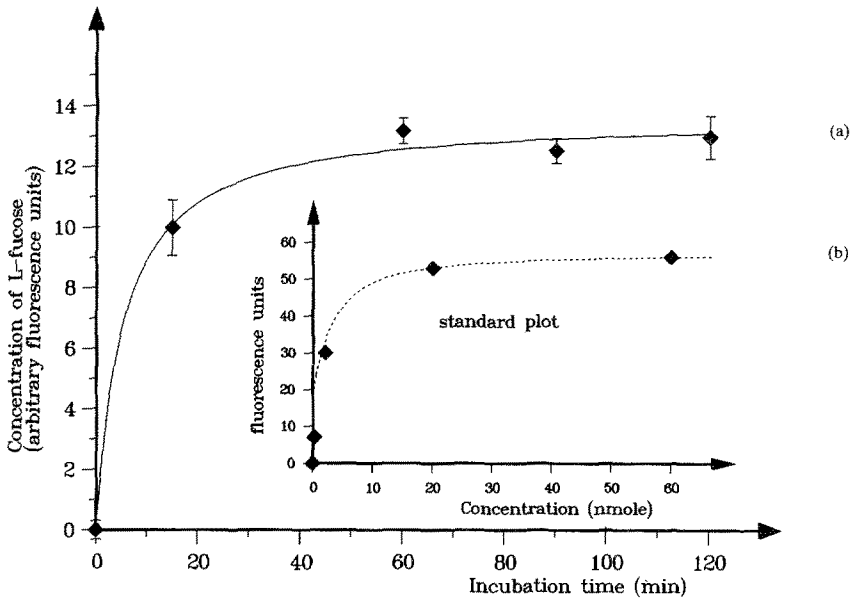

Figure 2. (a) Determination of L-fucose after acid hydrolysis of CU dialysis membranes. Membranes were hydrolysed with 0.1 M HCl at 90 °C for 15, 60, 90 and 120 min and free L-fucose was measured by the enzymatic fluorescence assay as stated in the Materials and methods. Fluorescence is in arbitrary units with a fluorescence of zero equal to that of double distilled water. Values are averages of quadruplicates (±SD). (b) A standard plot of fluorescence intensity versus L-fucose concentration.

Table 2. Concentration of L-fucose residues on dialysis membranes after acid hydrolysis.

Membrane	*0.1 M HCl*[a]	*Distilled water*[b]
Cuprophan	90.2 ± 5.0	5.6 ± 0.3
Cuprophan with fucosidase pretreatment	81.9 ± 4.0[c]	–
Hemophan	79.3 ± 3.6[c]	5.7 ± 0.8
AN-69	14.0 ± 2.1[c]	2.6 ± 0.5
Gambrane	5.7 ± 2.4[c]	1.9 ± 0.4

[a]Values were determined after hydrolysis of punched flat sheet dialysis membranes (6 mm i.d.) with 1 M HCl for 60 min at 90 °C. After neutralization, L-fucose was measured using an enzymatic fluorescence assay. Fluorescence of blanks (0.1 M HCl) was equal to that of pure double distilled water. See text for experimental details.
[b]Membranes were incubated with double distilled water in order to compare with acid hydrolysis.
[c]Results significantly differ from values for CU; $p < 0.01$, paired t-test.

and 79.3 pmol cm^{-2}) were found to be statistically significant ($p < 0.01$, $n = 8$, paired t-test). A significant difference (8.3 pmol cm^{-2}) was also obtained between the values for CU after acid hydrolysis and CU which was preincubated with α-L-fucosidase for 14 h prior to acid hydrolysis (81.9 pmol cm^{-2}; $p < 0.01$, $n = 8$). This reduction may account for the amount of L-fucose which is accessible to the enzyme.

In a further set of experiments, membranes were incubated with α-L-fucosidase for 14 h and the L-fucose of the supernatants was assayed. The blank values for these determinations were much higher compared to those of the acid treated samples so that the results have to be interpreted with some care. Nevertheless, 7.7 ± 3.3 pmol cm^{-2} ($n = 4$) was detected for CU but no fucose was detected with PC-PE. These results agree with the acid hydrolysis ± fucosidase results which suggested that 8 pmol L-fucose per cm^2 was released by the enzyme. Detection limit of the L-fucose assay was 2–3 pmol.

Identification of lectin accessible L-fucose

Competitive inhibition of peroxidase labelled UEA-I by unlabelled UEA-I and LTA To further determine whether the L-fucose residues on the surface of cellulose membranes were biologically accessible, we studied the binding of fucose-specific lectin to CU. UEA-I or LTA specifically bound to membrane whereas PHA (lectin without any specificity for L-fucose) did not.

Preincubation of CU membranes with UEA-I or LTA competitively inhibited the binding of a given amount of peroxidase labelled UEA-I lectin (Fig. 3). This inhibition was considerable for UEA-I, and to a smaller extent for LTA lectin. Differences between LTA and UEA-I are likely to be due to the different physical and chemical properties of the lectins, and in particular, to the different binding constants with fucose-containing ligands [12]. However, the differences between LTA and UEA-I compared with PHA are obvious.

Figure 4 shows electron microscopy photographs obtained after incubation of CU with biotin/avidin-gold

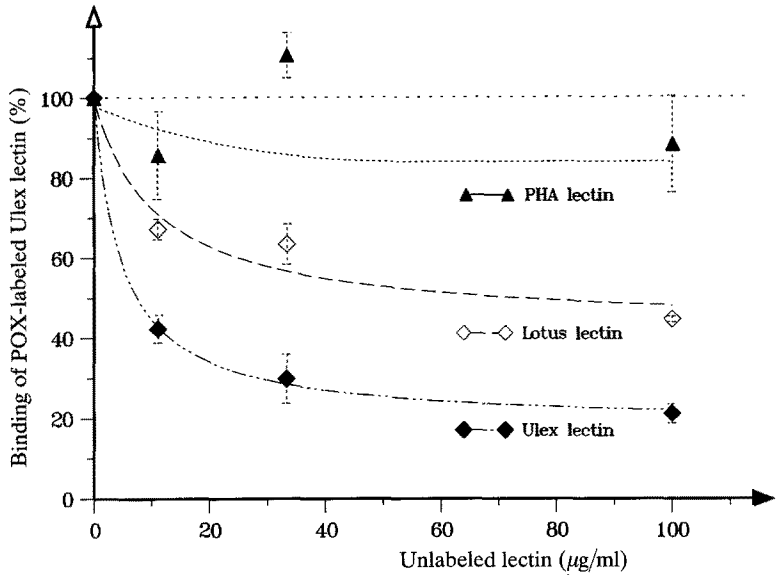

Figure 3. Inhibition of HRP-labelled UEA-I lectin binding to CU membranes by UEA-I and LTA lectins and a non-fucose specific lectin, PHA. Adsorption values are expressed as a percentage of uninhibited lectin binding. All data are given as means ± SEM (n = 4).

labelled LTA and PHA (see experimental for details). The images clearly demonstrate increased binding of LTA in contrast to PHA and the control.

The broader specificity of the LTA for fucose containing ligands [13], caused us to choose this protein for further analysis of lectin binding.

Detailed study of the binding of LTA to the CU membrane To analyse more precisely lectin binding, different concentrations of peroxidase labelled LTA were added to CU membranes. After repeated washing, specifically bound lectin was evaluated as shown in Fig. 5. The adsorption of lectin appeared saturable at a concentration of about 50 $\mu g\,ml^{-1}$ of added lectin. Binding could be competitively inhibited by incubation of the lectin with 5 mM L-fucose. The presence of L-fucose on the membrane is represented by the difference between saturation levels for normal lectin binding and those after L-fucose treatment.

Lectin binding was further analysed on these membranes after treatment with α-L-fucosidase. These values were similar to those for L-fucose inhibited binding, indicating that lectin and enzyme probably act on the same structures on the membrane.

L-Fucose was not detected on PC-PE and AN-69 membranes (results not shown). Small non-specific protein adsorptions for these materials were found; they may be due to different adsorption capacities of the membranes [14]. However, blocking of these sites could suppress this effect. A low affinity for detergent-lectin complexes may also explain the non-specific effects.

From the binding data the surface fucose was calculated using a reciprocal Scatchard analysis [15, 16]. The following assumptions were made: i) lectin and immobilized fucose react in a one-to-one ratio; ii) the separation of bound and free lectin is complete and the washing procedure does not dissociate the lectin-fucose complex; iii) the affinities of the lectin binding sites for the different isolectins of LTA are similar; iv) the system achieves equilibrium; v) lectin binding is completely inhibited in the presence of 5 mM monomer L-fucose; and vi) the peroxidase reaction is identical in the bound and free lectin.

The fucose content of fucosidase and fucose treated membranes were calculated as 2.4 ± 0.15 pmol fucose per cm^2 ($n = 4$) and 2.6 ± 0.25 pmol fucose per cm^2 ($n = 4$). Both values are very similar and indicate the presence of biologically accessible L-fucose on the surface of the CU polymer.

Discussion

Regenerated cellulose is a powerful and versatile polymer commonly used in commercial haemodialysers. However, clinical application of this material has revealed a number of problems with compatibility, which in part may be due to a complement-independent activation of leukocytes by contact with the dialysis membranes. In the absence of a complement, an increased release of inflammatory mediators was observed [9] as well as increased expression of β2-microglobulin mRNA after short contact of the cells with CU [7, 8]. These effects could be specifically inhibited by the monosaccharide

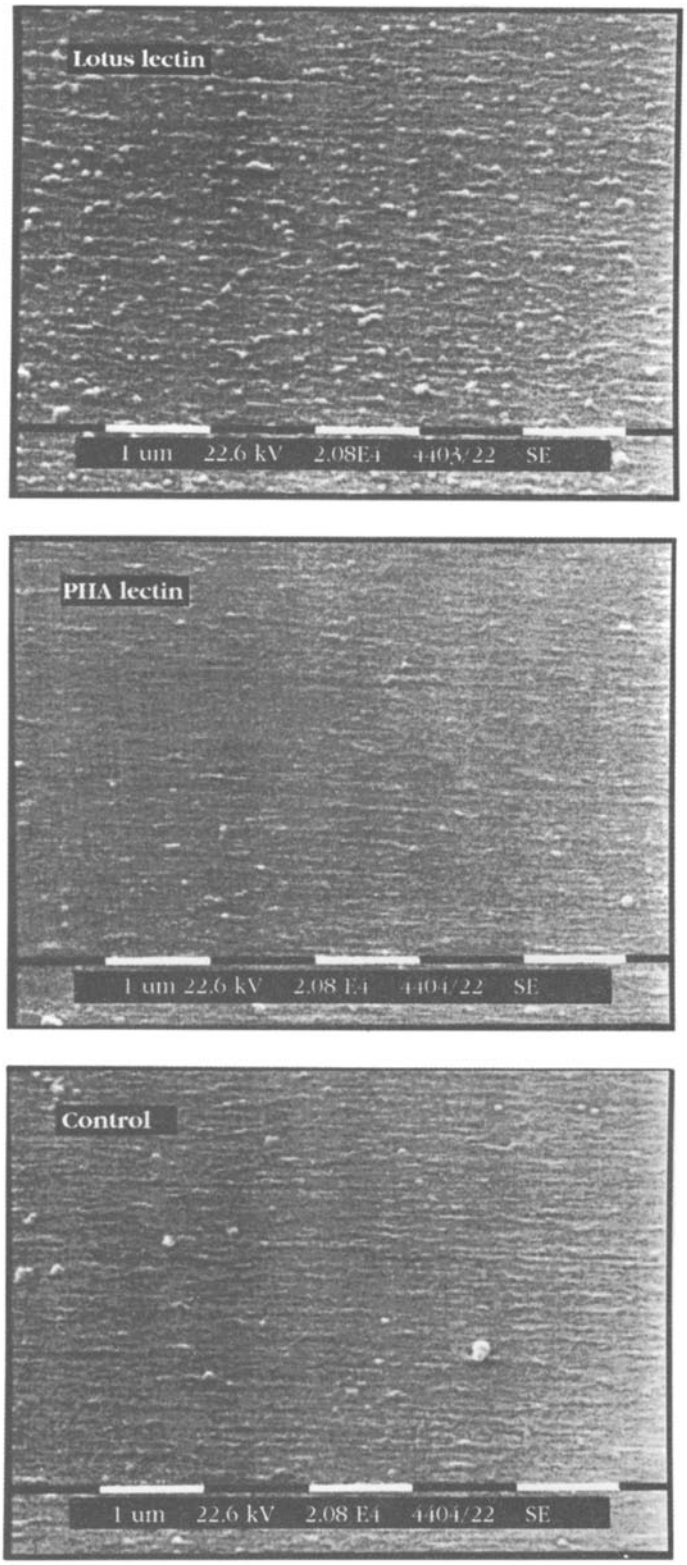

Figure 4. Electron-micrographs of CU membrane treated with LTA and detected by gold-labelling. The white spots indicate the presence of gold-labelled lectin. Homogeneously distributed patterns of globular structure can only be seen when LTA (top) was used as compared to the results obtained without LTA (bottom) or using PHA (middle) which does not bind to fucose.

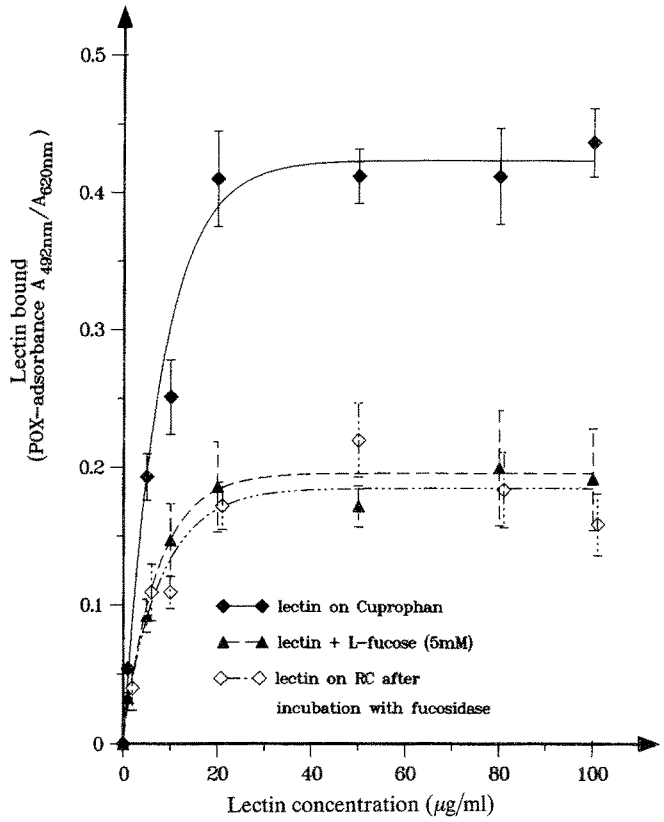

Figure 5. Adsorption isotherm of *Lotus tetragonolobus* lectin onto cellulosic Cuprophan. OD values are given as the result of the OPD assay for POX-labelled lectin (see Materials and methods). Background absorption at 620 nm was substracted. Linearity of the standards is given in the specific regions of free lectin concentration. Mean value and standard deviation (vertical bar) are given for each point ($n = 4$).

L-fucose, but not by its enantiomer or other monosaccharides. This leads to the hypothesis that membrane-associated fucose residues may participate in cell activation. Since L-fucose is present in naturally occurring cellulose, although in small concentrations [22], it is possible that it is retained during the manufacture of the dialysis membranes by the cupra-ammonium process.

We investigated commercially available flat sheet dialysis membranes for the presence of L-fucose by using a sensitive enzymatic fluorescence assay. The assay, which is based on a cycling reaction first developed by Lowry *et al.* [10], enables the detection of as little as 4 pmol L-fucose; it does not require extensive sample preparation or derivatization of the saccharide prior to analysis as do chromatographic techniques [17, 18]. The results of the fluorescence assay clearly demonstrate the presence of picomolar amounts of L-fucose on the CU and HE dialysis membranes, however, with the AN-69 and PC-PE membranes only small fluorescence signals were obtained, which may be due to contamination of the membranes with fucose-containing bacterial LPS. The

DEAE-modified cellulose HE membrane which is known for its better biocompatibility [19, 20] contains less L-fucose residues compared with CU. It is assumed that the chemical substitution of reactive OH-groups by DEAE also affects some fucose residues which in turn cannot be oxidized by the fucosedehydrogenase in the fluorescence assay. It may also be argued that these modified fucose residues are no longer susceptible to other fucose-specific enzymes or fucose-binding proteins.

L-fucose was also determined after enzymatic cleavage of the saccharide with α-L-fucosidase. Much less fucose was detected, due to the fact that only surface-associated L-fucose was removed and quantified.

It is known that the L-fucose dehydrogenase used in the fluorescence assay can also act on sugars other than L-fucose including D-arabinose, L-xylose and L-galactose, but at a much decreased rate [21]. However, they are probably of no importance in our analysis, because they are not common in cotton linters, the starting material for the production of cellulose membranes [22].

Binding of LTA and UEA-I to CU was studied to see whether L-fucose is able to act as ligand for these lectins. The lectins bound to CU and fucose-specificity was demonstrated: i) by the competitive inhibition of binding with L-fucose; ii) by reduced lectin binding after enzymatic removal of surface-associated L-fucose with α-L-fucosidase. Only nonspecific protein adsorption was found for the AN-69 and PC-PE membranes.

From the Scatchard analysis of lectin binding data, 2–3 pmol cm^{-2} of L-fucose were calculated to be present on CU, assumming univalent binding and also identical binding characteristics for the distinct isolectins. These calculated values are similar to those obtained by the fluorescence assay after enzymatic cleavage of membrane associated fucose.

This study provides additional evidence for our hypothesis that cellular contact activation involves L-fucose residues bound to the surface of cellulose dialysis membranes. L-Fucose is wide-spread in nature and usually located at the nonreducing terminus of sugar chains. This makes it accessible for enzymes, lectins or lectin-like fucose binding proteins which, for example, serve as cell surface receptors. L-Fucose participates in the interaction of macrophages with macrophage activating (MAG) and inhibitory factor (MIG) [23]; it is also part of a cellular LPS receptor involved in tumour cell killing [24]. Recent studies include the characterization of a mannose/fucose macrophage receptor [25] and the discovery of fucose containing oligosaccharidic ligands involved in selectin-mediated cell adhesion [26]. It is, therefore, conceivable that L-fucose residues on cellulose-based dialysis membranes could act as potential ligands for lectin-like leukocyte receptors participating in cellular contact activation. This hypothesis is in line with our recent studies [7, 9] in which cellular contact activation was mainly observed by CU membranes but not by the AN-69 and PC-PE polymers, which do not contain L-fucose.

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