Effect of Dextran 40 on Endothelial Binding and Vesicle Loading of Ferritin in Rabbit Aorta

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The effects of dextran 40 on endothelial binding and vesicle loading of cationized (CF, pI > 9.0) and anionic (AF, pI = 3.8 to 4.2) ferritins were investigated in the rabbit aorta. After two minutes of in situ perfusion, both ferritin species entered luminal vesicles and bound to the luminal endothelial membrane with a high surface density at vesicle necks. With CF, the presence of 5% dextran 40 did not change the fraction of vesicles loaded, the number of particles per vesicle, or the average density of particles bound to the luminal membrane, but did reduce the average density of particles adhering to vesicle necks. With AF, dextran 40 increased the percentage of vesicles filled, the average number of particles per loaded vesicle, the membrane particle density, the percentage of loaded vesicle necks, and the average density of particles adhering to vesicle necks. Application of gel chromatography and electrophoresis and determination of carbohydrates in the ferritin and dextran perfusates by the anthrone method after precipitation of the ferritin demonstrated that the dextran and ferritin did not conjugate. We hypothesize that the dextran binds to the endothelial membrane and increases its affinity to AF possibly by shielding negatively charged sites and that it binds in such a way as to offer little steric hindrance to AF particles entering vesicles. (Arteriosclerosis 8:140–146, March/April 1988)

For several years dextran 40 and 70 have been successfully used prophylactically by intravenous infusion in patients who have a high risk of thrombosis.1–5 The dextran decreases platelet aggregation and thrombus stability by interacting with the protein, factor VIII, which is bound to platelets. Dextran has also been shown to increase vascular permeability to polyamionic plasma proteins such as albumin.6–8 Such alterations in transport could cause complications in these patients, such as changes in intercompartamental protein distribution, water balance, and possibly an increased incidence of atherosclerosis. Elucidation of the mechanism by which dextran increases macromolecular permeability may suggest methods by which such increased permeability can be reduced.

Previous studies using the isolated perfused rabbit ear6,7 and rat hindquarters8,9 demonstrated that neutral dextran increased vascular permeability to albumin and that this effect increased with dextran concentration. Abee9,10 concluded that the dextran molecules interact with macromolecular exchange channels, making them more accessible to the penetration of plasma protein molecules. Haraldsson et al.9 additionally speculated that the adherent dextran molecules also reduce the negative charge of the capillary wall. In this situation negative macromolecules such as albumin would pass more easily through the endothelium.

In this study we investigated Haraldsson’s charge hypothesis by determining the effect of dextran 40 on endothelial transport of cationized and anionic ferritins. Ferritin has the advantage that it can be viewed with the electron microscope, so the effects of dextran on ferritin uptake can be examined at an ultrastructural level. The experiments described in this study were performed on in situ preparations of rabbit thoracic aorta because we are experienced in using this preparation to study ferritin uptake and already have information on the effects of molecular charge10,11 and the presence of plasma proteins12 on endothelial uptake of ferritin.

Methods

Animals

The experiments were performed on 24 New Zealand white rabbits weighing 2.5 to 3.5 kg each.

Ferritin

Cationized ferritin (CF) and anionic ferritin (AF) were obtained from Sigma Chemical Company (St. Louis, Missouri). By isoelectric focusing on polyacrylamide gels, the isoelectric points (pI) were found to be >9.0 and 3.8 to 4.2, respectively. The ferritin was used in Tyrode’s solution, pH 7.4, CF at concentrations ranging from 0.05 to 2.0 mg/ml and AF, from 25 to 200 mg/ml.

Tests of Conjugation of Ferritins with Dextran T40

Five percent (wt/vol) neutral dextran T40 (Pharmacia Fine Chemicals, Piscataway, New Jersey) was dissolved

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in the ferritin solutions for some experiments. To determine whether there was any binding of dextran to the ferritin particles, three types of tests were carried out: determina-
tion of the carbohydrate content in the ferritin and dextran perfusates by the anthrone method after precipitating the ferritin, gel chromatography, and electrophoresis, as de-
scribed below.

**Anthrone Method**

Four paired samples of CF and AF solutions (1 mg/dl) were made and dextran T40 was added to each pair to give concentrations of 0, 1, 3, and 5 mg/dl. The solutions were then centrifuged at 45 000 rpm for 3 hours. The optical densities of the supernatants were recorded on a spectrophotometer Model B (National Technical Laboratories, South Pasadena, California) at a wavelength of 375 nm against a water blank to ensure that all the ferritin had precipitated. The anthrone reaction was performed on six samples of each of the four paired supernatants, on dis-
tilled water (serving as a blank), and on paired standard dextran T40 solutions (1, 3, and 5 mg/dl). Optical densities (OD) of the anthrone reaction product of the supernatants at 650 nm were compared with those of the standard solutions.

If the OD of a supernatant reaction product was signifi-
cantly lower than that of the dextran standard of the same
initial concentration, this would indicate that some dextran had bound to the ferritin and been precipitated. To avoid spurious results due to contamination, we used new tubes for each test.

One disadvantage of this test is that dextran concentra-
tions must be kept down to 6 mg/dl. This means that to keep the CF/dextran ratio similar to that used in experi-
ments, a CF concentration of 0.03 mg/dl is required. If dextran binds to CF, the amount removed from the supernatant may be too small to detect. For this reason gel chromatography and electrophoresis were carried out, as described in the next two paragraphs.

**Gel Chromatography**

A 1 cm diameter column was packed with Sepharose 6B (Pharmacia) to a height of 30 cm, and was equilibrated with 0.02 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4). Samples of pure ferritin and ferritin with dextran were applied, keeping the ferritin/dextran ratios the same as those used in experiments. Two ml eluant aliquots were collected from the column and the ODs were recorded by use of a spectrophotometer at a wavelength of 375 nm. The plots of cumulative volume versus OD which were obtained were compared for solutions with and without dextran.

**Electrophoresis**

Electrophoresis of CF and AF with and without dextran T40 was carried out using a barbitone buffer (0.05 to 0.07 M) (pH 8.6) in a Beckman microzone cell, Model R-101 (Beckman Instruments Incorporated, Fullerton, California) with a cellulose acetate membrane support me-
dium. The electrophoretograms were stained with a 0.2% solution of Ponceau S in 3% aqueous trichloroacetic acid for 10 minutes and were washed in 5% aqueous acetic acid before drying.

**Surgery and In Situ Perfusion**

The rabbits were anesthetized intravenously with sodium pentobarbital (30 mg/kg). The trachea was cannulat-
ed, and respiration was maintained with a ventilator. The aorta was cannulated in situ as described in a previous publication. Briefly, a catheter tubing was inserted into the iliac bifurcation of the aorta and advanced until its tip was just proximal to the origin of the celiac artery where it was tied securely in position. Simultaneously, the aorta was ligated distal to the origin of the innominate artery, and the catheter tubing was connected to a reservoir of Ty-
rode's solution which was air-pressure at 50 mm Hg. A 16-gauge butterfly needle catheter which was also con-
ected to the reservoir was inserted into the aorta just distal to the ligation and was secured. The catheter tubing was then disconnected from the reservoir and opened to the atmosphere to serve as an outlet. The animal was sacrificed with an overdose of anesthetic and the cannulat-
ed segment was perfused with Tyrode's solution with 5% dextran T40 (38°C) for 1 min at a flow-rate of 5 to 6 ml/min. The Tyrode's solution was replaced by the ferritin-
dextran solution (38°C) at 50 mm Hg. This pressure was chosen because it was sufficient to prevent aortic collapse but low enough to keep loss of perfusate through the inter-
costals to an acceptable level. After 2 minutes, the ferritin solution was replaced by Karnovsky's fixative at 50 mm Hg and 4°C. The fixative was also applied to the outer surface of the aorta and renewed every 20 minutes for 1 hour. The vessel was then excised, any possibly damaged areas near the cannula tips were discarded, and the remainder was cut into segments approximately 1 cm long.

The segments were postfixed in 1% sodium cacodylate buffered OsO4 at 4°C for 1 hour, they were stained for 30 minutes with 1% uranyl acetate, they were dehydrated in ascending series of ethanol, and were embedded in Spurr's resin for electron microscopy. Sections were cut perpendicular to the intimal surface with a diamond knife on a MTZ-B Sorval Ultramicrotome (DuPont Instruments) and they were stained with uranyl acetate, lead citrate, and bismuth subnitrate. The sections were examined with a ZEISS EMSS electron microscope.

**Estimation of Plasmalemmal and Vesicular Labelling with Ferritin**

From 50 to 60 sections of thickness 600 to 800 Å, as judged by their silver interference color, were cut transverse from two different areas of each vessel and were examined in the electron microscope. For the quantitation of plasmalemmal labelling, 20 prints with a final magnification of 80 000 x were taken from each aorta. The length of the endothelial plasmalemmal membrane was measured, and the number of attached ferritin particles were counted for each print to obtain the particle density along the mem-
brane (PD). We used a Hipad Digitizer (Houston Instru-
mment, Austin, Texas) linked to a PDP-11 computer (Digital Equipment Corporation, Maynard, Massachusetts). Areas
of cellular overlap (flaps) were not included in these measurements. The total number of luminal vesicles with labelled necks and the total number of ferritin particles adhering to vesicle necks were counted to obtain the average number of particles per vesicle neck for each print. This figure was then divided by the mean vesicle neck diameter, 43.0 ± 0.6 nm (n = 1000), to give the average number of particles per micron at vesicle necks (PDv). For measurement purposes, we define the size of the vesicle neck as the shortest distance between opposite sides of the invagination. Only the necks of fully formed vesicles were included in the PDv measurements. When particles at a given vesicle neck were counted, only those contained within the space between opposite sides of the invagination were included. The mean values of PD and PDv for each experiment were obtained by averaging the individual print values.

We assessed the labelling of luminal vesicles by determining the total number of vesicles counted (Nv), the number of vesicles labelled with ferritin (Nl), and the total number of ferritin particles present within the vesicles, not including those bound to vesicle diaphragms (F). True values for F could not be obtained from the electron micrographs simply by counting the number of particles within the vesicles because the mean section thickness was comparable to the mean vesicle diameter and so a fraction of most vesicles seen in the electron micrographs lay outside the section. Previous calculations demonstrate that, on the average, the fraction of vesicle volume which lies within sections of 700 Å thickness is 0.8125.14 Final values for F were therefore calculated by dividing the apparent values by this factor. From these results, we calculated Nl/Nv, which is the fraction of vesicles in a given population containing any ferritin particles, and F/Nl, which is the mean number of particles per labelled vesicle. Over 200 vesicles were counted for each vessel and in a given cell only every fifth luminal vesicle was included in the count to maximize the number of cells sampled. All the measurements made depend on the section thickness and it is assumed that the effects of variation in thickness within the range 600 to 800 Å average out when the number of sections sampled is sufficiently large.

Results

Tests of Conjugation of Ferritins and Dextran T40

Anthrone Reaction

The optical densities of the ferritin-dextran supernatants after the anthrone reaction were not significantly different from those of corresponding standard solutions of the same initial dextran concentration (Table 1). This indicates that there was no detectable binding of dextran to CF or AF under our experimental conditions.

Gel Chromatography

The presence of dextran in solutions of CF (Figure 1A) and AF (Figure 1B) did not change the elution volume profile. This indicates that dextran does not change the hydrodynamic radius of the ferritin particles, and is further evidence of the lack of binding of dextran to ferritin.

Electrophoresis

Electrophoresis (Figure 2) demonstrated that the electrophoretic mobilities of CF and AF were not changed by the presence of dextran T40.

Effect of Dextran T40 on Endothelial Binding and Vesicle Loading

Statistical comparisons between results with and without dextran T40 were made in four experiments using 0.5 mg/ml CF, and in four experiments using 0.1 mg/ml AF. The data points for other concentrations of CF and AF (one or two at each concentration) are included on the graphs in Figures 3 and 4 to illustrate whether results with and without dextran T40 follow the same general trend. In Figures 3 to 5 each error bar represents the standard deviation of the mean value averaged over four experiments, and data points without error bars represent single experiments.

Cationized Ferritin

Data from the present experiments using CF with dextran T40 were compared with those from previous studies using CF alone10,11 (Table 2). These previous studies include six experiments using 0.5 mg/ml CF and a total of 10 experiments using other concentrations. Values of Nl/Nv (mean ± SD) with and without dextran were 90% ± 2% (n = 430) and 91% ± 6% (n = 720), respectively; these values were not significantly different, and both sets of data followed the same trend at other concentrations (Figure 3). Similar results were found for F/Nl; values with and without dextran were 8.6 ± 4.7 (n = 390) and 9.0 ± 4.8 (n = 650), respectively, and were not significantly different. Binding to the endothelial plasmalemma was not affected by dextran; the values were 132 ± 30 μm⁻¹ (n = 40) with dextran and 133 ± 43 μm⁻¹ (n = 65) without dextran. Both sets of data followed a similar isotherm, levelling off at a PD of 180 μm⁻¹. At a CF concentration of 0.5 mg/ml, almost all vesicle necks were labelled, whether
Figure 1. Elution profiles of: A. 2 mg/ml cationized ferritin (CF) (●) and 2 mg/ml CF with 1.9 mg/ml dextran T40 (○) and B. 2 mg anionic ferritin (AF) (●) and 2 mg AF with 0.38 mg/ml dextran T40 (○) after gel chromatography.

Figure 2. Electrophoresis of ferritin and dextran solutions. This figure is a tracing of the original electrophorogram. The left side of the membrane was connected to the negative terminal and right side, to the positive terminal. Solutions of cationized ferritin (CF) (a) and anionic ferritin (AF) (b) were initially placed in line with the origin (○). 1. CF and dextran T40 in Tyrode's solution, 2. CF in Tyrode's solution, 3. AF and dextran T40 in Tyrode's solution, and 4. AF in Tyrode's solution.

Figure 3. The relationship between mean number of vesicles labelled with ferritin + total number of vesicles counted (Nv/Nv) and cationized ferritin (CF) concentration without dextran (●—●) and in the presence of 5% dextran T40 (○—○). The curves were fitted by eye. The error bars represent SD.

Figure 4. The relationship between mean number of vesicles labelled with ferritin + total number of vesicles counted (Nv/Nv) and anionic ferritin (AF) concentration in the presence of 5% dextran T40 (○) and without dextran (●). The error bars represent SD.

Figure 5. Particle density at vesicle neck + particle density (PDv/PD) as a function of anionic ferritin (AF) concentration in the presence of 5% dextran T40. Each point represents one experiment.
dextran was present or not. Binding to vesicle necks was significantly greater without dextran, \( p < 0.005 \), (244 ± 84, \( n = 270 \)) than with dextran (170 ± 52, \( n = 50 \)), but this trend was not seen at all other CF concentrations. CF particles showed a marked predilection for vesicle necks compared to the plasmalemmal membrane at low concentrations (0.1 mg/ml) in the presence of dextran; PDV was almost 10-fold greater than PD. This trend has also been noted in the absence of dextran.\(^{10,11}\)

**Anionic Ferritin**

Data from experiments using AF with dextran were compared (Table 3) with those from control experiments using AF alone (five experiments from this study combined with four from a previous study\(^{11}\)). Values of \( N_L/N_T \) for AF concentration 0.1 g/ml were significantly greater (\( p < 0.005 \)) with dextran (82% ± 1%, \( n = 270 \)) than without dextran (68% ± 5%, \( n = 900 \)). This trend was also seen at other AF concentrations. There was a wide variation in \( N_L/N_T \) between experiments when AF was used without dextran, and it was not possible to ascertain whether there was a plateau \( N_L/N_T \) value (Figure 4). When dextran was present, \( N_L/N_T \) levelled off at 80%. Values of \( F/N_L \) at 0.1 g/ml AF were significantly greater (\( p < 0.005 \)) with dextran (4.4 ± 3.1, \( n = 221 \)) than without (2.8 ± 1.8, \( n = 620 \)), and this trend was also seen at other AF concentrations.

**Discussion**

The increased endothelial binding of AF in the presence of dextran 40 suggests that the adsorption of dextran 40 to the endothelial surface decreases the surface zeta potential. It has previously been shown that dextran 40 has the opposite effect on red blood cells; in this case the zeta potential is increased.\(^{15}\) To explain the observation on red blood cells, it has been hypothesized that the dextran reduced the space available for positive counter ions in the solution to shield the negative charge on the cell surface.\(^{16}\) However, no direct comparison can be made between the red blood cell and endothelial surfaces because the former consists mainly of sialic acid, whereas the latter also includes heparin-related mucopolysaccharides.\(^{17,18}\) It is possible that in the case of the endothelium, the tendency

### Table 2. Labelling of Aortic Endothelium with Cationized Ferritin with and without 5% Dextran T40

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<th>Surface labelling (μm⁻¹)</th>
<th>Vesicular labelling</th>
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<tr>
<td></td>
<td>PD</td>
<td>PDᵥ</td>
</tr>
<tr>
<td>CF</td>
<td>133 ± 43 (n = 65)</td>
<td>244 ± 84 (n = 270)</td>
</tr>
<tr>
<td>CF + dextran T40</td>
<td>132 ± 30 (n = 40)</td>
<td>170 ± 54 (n = 50)</td>
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Values are means ± SD. The luminal concentration of cationized ferritin was 0.5 mg/ml. PD = particle density; PDᵥ = particle density at vesicle neck; CF = cationized ferritin; \( N_L \) = no. of vesicles labelled with ferritin; \( N_T \) = total no. of vesicles counted; \( F \) = total no. of ferritin particles within vesicles.

### Table 3. Labelling of Aortic Endothelium with Anionic Ferritin with and without 5% Dextran T40

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<tr>
<td></td>
<td>PD</td>
<td>PDᵥ</td>
</tr>
<tr>
<td>AF</td>
<td>17 ± 6 (n = 70)</td>
<td>82 ± 26 (n = 80)</td>
</tr>
<tr>
<td>AF + dextran T40</td>
<td>88 ± 34 (n = 34)</td>
<td>120 ± 27 (n = 80)</td>
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Values are means ± SD. The luminal concentration of anionic ferritin (AF) was 0.1 g/ml. See legend of Table 2 for explanation of abbreviations.
of the dextran to shield the negatively charged carbohydrate chains (thus reducing the zeta potential) has a stronger effect than the exclusion of positively charged ions from near the cell surface.

In this study it was necessary to use protein-free perfusates because cationized ferritin complexes with anionic plasma proteins. Our previous experiments\(^1\) show that perfusion of the rabbit aorta for 1 minute with protein-free Tyrode's solution removes some of the plasma proteins normally adherent to the endothelial glycocalyx and increases the net negative surface charge on the endothelium. This study therefore concerns the interaction of dextran with an endothelial glycocalyx that has been partially depleted of bound plasma proteins. However, our observations are still relevant to vessels perfused with plasma because the presence of plasma proteins does not prevent dextran from binding to the glycocalyx\(^2\) and does not alter the effect of dextran on macromolecular permeability.\(^3\)

The finding that the presence of dextran increased access of AF particles to vesicle cavities implies that the dextran molecules which are absorbed to the luminal membrane do not offer significant steric hindrance to vesicle entry. Several studies have shown that albumin\(^1\) and other plasma proteins\(^1\) stericly hinder the vesicle entrance of AF particles. Since the molecular weight of dextran 40 is only 40% less than that of albumin, its lack of steric hindrance regarding ferritin seems surprising at first sight. However, unlike the albumin molecule, which is basically spherical in shape, dextran molecules are rod-shaped with a marked axial asymmetry.\(^2\) A previous study on the dog aorta\(^2\) suggests that dextran 40 molecules adsorb to the endothelial surface end-on; the concentration of radioactive dextran on the endothelial surface was too high to be accounted for by a layer of dextran molecules absorbed parallel to the surface. If the molecules are sufficiently far apart, such a configuration may explain the lack of steric hindrance to AF vesicle entry that we observed in this study. Dextran 75 molecules have been shown to enter capillary endothelial vesicles,\(^2\) and it is possible that the dextran molecules within the vesicles shield the negative charges on the internal vesicular surface to a similar extent as at the vesicle neck. This would result in comparable electrostatic attractive forces for AF particles within the vesicular cavity and at the vesicle neck and would explain why AF vesicular entrance is not hindered by preferential electrostatic attraction to the vesicle neck.

A surprising result of this study was that the presence of dextran did not adversely affect plasmalemmal binding and vesicle entry of CF particles. A possible explanation is that the electrostatic interaction between CF and the anionic endothelial binding site is so strong that it is not significantly reduced by the shielding effect of the bound dextran molecules, whereas the repulsive force between AF and the anionic sites is relatively weak and can be more effectively shielded. Our study shows that dextran 40 increases plasmalemmal binding and vesicle entry of AF particles in the aorta, and the results with CF suggest that this occurs by shielding of the anionic charges on the glycocalyx by the dextran molecules. If transcytosis or formation of transient vesicle channels provide thoroughfares for plasma proteins through the endothelium, it is likely that permeability to these macromolecules will be increased by similar mechanisms. One way in which this effect might be removed when administering dextrans to patients with a high risk of thrombosis would be to use sulfated dextrans because they would not decrease the vessel surface negative zeta potential.

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