NONRANDOM DISTRIBUTION OF SIALIC ACID
OVER THE CELL SURFACE
OF BRISTLE-COATED ENDOCYTIC VESICLES
OF THE SINUSOIDAL ENDOTHELIUM CELLS

PETER P. H. DE BRUYN, SONIA MICHELSON, and ROBERT P. BECKER

From the Department of Anatomy, University of Chicago, Chicago, Illinois 60637. Dr. Becker's present address is the Department of Anatomy, College of Medicine, University of Illinois, Chicago, Illinois 60680.

ABSTRACT

Previous studies with protein tracers have shown that the luminal surface of the vascular endothelium of the bone marrow is endocytic. The endocytosis occurs through the formation of large bristle-coated vesicles (LCV). The anionic charge distribution in this process was examined at the luminal surface of the endothelial cell. At pH 1.8, colloidal iron (CI), native ferritin, and polycationic ferritin (PCF) are bound by the luminal surface of the endothelial cell, but not at the sites of LCV formation. PCF used over a pH range of 1.8-7.2 (CI is unstable at higher pH levels) revealed LCV binding of this agent in increasing manner from pH 3.5 upwards. PCF binding at low pH (1.8) at the endothelial cell surface was markedly reduced by neuraminidase. Neuraminidase did not reduce PCF binding by the endothelial cell surface nor by the LCV at higher pH levels.

It is concluded that the luminal surface of the endothelial cell has exposed sialic acid groups which are absent or significantly diminished at endocytic sites. The free surface of the endothelial cells as well as the sites of endocytosis have, in addition, anionic material with a pKₐ higher than that of sialic acid (pKₐ 2.6). These anionic materials may be different at the sites of endocytosis as compared to those present at the free cell surface.

KEY WORDS  endocytosis · cell surface · coated vesicle · endothelium · bone marrow · sialic acid

Studies with intravascularly administered tracers (carbon, native ferritin, horseradish peroxidase) have shown that the endothelial cell of the sinu-
soids of the bone marrow is endocytic (2, 10, 22, 23). In the initial stages of endocytosis, blood-
borne tracers become affixed to bristle-coated indentations at the luminal surface of the endothelial cell. Subsequently, these indentations become pinched off as large bristle-coated vesicles (LCV) enclosing the tracers and appearing isolated in the

1 These large bristle-coated vesicles (1,000-2,100 Å in diameter; mean diameter: 1,400 Å), which are derived from the plasmalemma, must be held to be distinct from the small bristle-coated vesicles (300-500 Å in diameter; mean diameter: 400 Å), which are probably derived from the Golgi cisternae (10, 15). It should also be noted that the bristle-free so-called micropinocytotic caveolae (600-900 Å in diameter; mean diameter: 800 Å), derived from endothelial plasmalemma in other vessels, do not occur in the bone marrow sinusoids (10).
cytoplasm of the endothelial cell. The further processing of tracers endocytosed in this way has been described previously (10), and does not concern us here. This study reports on changes demonstrable at the endothelial cell surface at sites of endocytosis, as revealed by the differential binding to the surface of fixed sinusoidal endothelial cells by a positively charged colloidal iron sol, a polycationic (high isoelectric point) ferritin (PCF), native (low isoelectric point) ferritin, and phosphotungstic acid (PTA).

MATERIALS AND METHODS

Male Long/Evans rats weighing 100–120 g were used throughout.

Colloidal Iron (CI)

Bone marrow was fixed by immersion for 2–4 h in a double aldehyde fixative consisting of 2% glutaraldehyde, 1.5% paraformaldehyde in 0.08 M cacodylate buffer, pH 7.2, to which 0.15% CaCl had been added. The fixation procedures as well as the method for removing of bone marrow with minimal damage to the delicate sinusoidal endothelium have been reported previously (2, 3, 12). After fixation, the bone marrow blocks were placed in 0.08 M cacodylate buffer, pH 7.2, to which 5% sucrose was added. 30–40-μm thick sections were cut with a Vibratome (Oxford Laboratories Inc., Foster City, Calif.), with the same cacodylate buffer-sucrose solution in the trough. These sections were rinsed in 20% acetic acid (pH 1.8) and placed for 2 h to overnight in a positively charged CI sol which was prepared according to the method of Rinehart and Abul-Haj (42). Before use, the CI suspension was dialyzed 2–3 days against distilled water, which itself was changed two to three times daily. Before use, 3 parts of the dialyzed suspension was diluted with 1 part of glacial acetic acid, resulting in a suspension of pH 1.8. This suspension, prepared in this manner, was chosen because it readily penetrates cells and gives positive reactions in intracellular components (19, 24, 52; see also Fig. 3). The particle size of suspensions appears to vary according to the method of preparation and is given variously as being ~30 Å in diameter (7) for the Mowry (28) colloidal iron, ~50–150 Å in diameter (51) for the sol prepared according to Gasic et al. (16). Our own preparation, based on measurements with a Joyce-Loebl III C densitometer (Joyce, Loebl and Co., Gateshead-on-Tyne, England) of particles in thin sections, yielded figures for the diameter of the particulate ranging from 53 to 122 Å, with a mean of 82 Å (number of observations n = 20).

After the treatment with CI, the Vibratome sections were rinsed in 20% acetic acid, followed by distilled water, and postfixed in an aqueous solution of 2% OsO4 for 2 h. The sections were dehydrated in graded ethanol and embedded in Epon 812 contained in BEEM capsules (Better Equipment for Electron Microscopy, Inc., New York), taking care that the sections were in a flat position on the bottom of the capsules.

Polycationic Ferritin

Polycationic ferritin (batch numbers CF 5, 10, 12, 14), prepared according to Danon et al. (8), was obtained from Miles Laboratories Inc., Elkhart, Ind., and was used for staining the endothelial cell surface at pH levels ranging from 1.8 to 7.2. No differences in results were obtained with the various batch numbers used. PCF (mean diameter: 113 Å; n = 25; measured in thin sections with a Joyce-Loebl III C densitometer) is comparable in diameter to native ferritin, viz., 110–112 Å for the air-dried crystals (20) and does not penetrate beyond the surfaces of the Vibratome sections. Therefore, a procedure for exposing sinusoidal endothelial surfaces to the PCF was adapted that was different from that used for CI. By means of a small rotating saw, the femur was opened by cutting a 3 × 1-mm slot in the shaft of the bone. After removing the epiphyses, the bone was immersed in the double aldehyde fixative for 2 h. The bone marrow was then removed as an intact pencil and fixed further for 2 h in the same fixative. The marrow pencil was cut lengthwise by means of small scalpel to expose and open the large central sinusoid (9). The blood clot contained in the sinusoid was removed by means of a fine forceps, exposing the luminal surface of the large central sinusoid. The resulting preparation is illustrated in Fig. 1. The half-pencil of marrow was then cut crosswise into blocks 2–3 mm long, which were in turn left in cacodylate buffer overnight.

For treatment at pH 1.8, PCF was diluted by adding 5 parts of 20% acetic acid to 1 part of PCF. For pH levels ranging from 2.5 to 3.5, 1 part of PCF was diluted with 5 parts of distilled water, the pH of which was adjusted with acetic acid. For higher pH levels, 1 part of PCF was diluted with 0.2 M acetate buffer, whereas near the neutral point 0.05 M barbital buffer was used. In this way, PCF solutions with the following pH levels were obtained: 1.8, 2.5, 3.5, 5.5, and 7.2. Blocks were exposed to PCF for 30–45 min. Before and after treatment with PCF, marrow blocks were thoroughly rinsed with the solutions of the corresponding pH levels minus the PCF. After staining, the blocks were rinsed in distilled water, osmicated by immersion in 25% aqueous OsO4, dehydrated and embedded in Epon 812 contained in flat silicone rubber molds, and oriented in such a way that sections crosswise to the long axis of the large central sinusoid could be conveniently obtained. Thin sections were lightly stained with lead and examined with an RCA EMU3G microscope.

Native Ferritin

Native horse spleen ferritin, twice crystallized and containing 25 μg of cadmium/ml, was obtained from Miles Laboratories Inc. A similar pH series, and in the
FIGURE 1 Scanning electron micrograph of rat bone marrow with the luminal surface of the large central sinusoid exposed. Numerous smaller sinusoids open into the large central sinusoid. Material prepared for scanning electron microscopy as described previously (7). × 100.

same dilutions as used for PCF, was used for treatment of tissue blocks with native ferritin.

Phosphotungstic Acid

Bone marrow was fixed as described above for staining with CI and PCF and embedded in glycol methacrylate, as described by Leduc and Bernhard (26). The method for staining of glycol methacrylate thin sections with PTA has been described previously (11).

Neuraminidase

Vibrio cholerae neuraminidase in sodium acetate buffer (0.05 M, pH 5.5, with 9 mg of sodium chloride and 1 mg of calcium chloride per ml added) was obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N.J. as a purified enzyme preparation and contained 500 U/ml. Neuraminidase preparations from this source have been checked for proteolytic activity by Haydon and Seaman (21) and by Bennett and Bondareff (4) by means of a method developed by Kuettnner et al. (25), and were found by them to be of a high degree of purity and free of contaminating protease activity. Before treatment with neuraminidase, blocks fixed and prepared as described for PCF were washed overnight in sodium acetate buffer, the same as that containing the neuraminidase. Marrow blocks were incubated for 20 h in the undiluted Behring Diagnostics neuraminidase solution at 37°C, with a change to a fresh incubating solution of neuraminidase after ~10 h of incubation. After a rinse in the sodium acetate buffer, the blocks were treated with PCF at different pH levels, as described above.

Control incubations consisted of incubating marrow blocks in the sodium acetate buffer vehicles without neuraminidase or in sodium acetate buffer containing freeze-denatured neuraminidase.

RESULTS

Colloidal Iron, pH 1.8

Colloidal iron readily penetrated the 30–40 μm thick Vibratome sections. The plasma membranes of the endothelial cells of the sinusoidal blood vessels, as well as the surface of the intra- and extravascular leukocytes and erythrocytes, bound CI in a uniform and evenly distributed fashion (Figs. 2 and 3), except at sites of LCV formation (Fig. 2). The membranes of LCV, whether open
FIGURE 2  Colloidal iron treatment at pH 1.8. The luminal endothelial cell surface binds the colloidal iron; the sites of LCV formation (arrowheads) do not. Note the colloidal iron binding by the surface of the erythrocyte in the upper right corner. Unstained. × 47,000.

FIGURE 3  Colloidal iron treatment at pH 1.8. Two immature leukocytes (WBC) and one macrophage (M). The surfaces of the blood cells bind colloidal iron; that of the macrophage binds none or very little. Note the intracellular colloidal iron binding by the granules of the leukocytes and the lysosomal bodies of the macrophage. Unstained. × 20,000.
to the vascular lumen or occurring isolated in the endothelial cells, consistently failed to react with the CI.

Often, some flocculent material could be seen at localized sites on the luminal surface of endothelial cells. Although this material was present in varying degree at sites of LCV formation, an increase in LCV size was usually attended by an increase in the amount of this material. Shallow, bristle-coated indentations had only little such material. When present, this material was not found to interfere with CI binding or the later described PCF binding at the cell surface.

Regarding the lack of staining of LCVs, it is of interest to note that the extravascular macrophages show a distinctly lesser degree of CI binding than the blood cells in the marrow (Fig. 3). In agreement with previous experiences, it was noted that the bristle coating was sometimes poorly preserved after the exposure of the tissue to low pH levels (15).

**Polycationic Ferritin**

Polycationic ferritin at pH 1.8 labeled the luminal endothelial cell membranes in an evenly distributed fashion, except for sites of LCV formation. In this respect, the staining pattern was very similar to that of CI at pH 1.8 (Figs. 4 and 5). LCVs, even in the early stages of formation, wherein the plasma membrane was barely indented (Fig. 4), consistently lacked binding with PCF. At pH 2.5 (Fig. 6), PCF binding was much the same as observed at pH 1.8 (Fig. 5); only occasionally was a particle found in the cavity of a LCV indentation. From pH 3.5 upward, both the generalized binding to the luminal surface of the endothelial cell and the binding at sites of LCV formation increased with increasing pH (Figs. 7–9.) For unknown reasons, the PCF binding at the LCV sites appeared in some instances to not be direct to the cell surface. At the higher pH levels examined, PCF particles had at localized places a tendency to aggregate (Figs. 8 and 9).

Since it might be considered that the negative results of PCF binding to LCV at low pH was due to the removal of a substance by the acid treatment, some material was exposed to 20% acetic acid (pH 1.8), followed by a wash in barbital buffer, pH 7.1, and then treated with PCF in the same barbital buffer. Upon examination, it was found that the degree of PCF binding to LCV at pH 7.1 did not differ from that present without acid pretreatment. This result excludes the possibility that the lack of LCV staining at low pH was the result of extraction of a material which binds PCF at high pH.

**Native Ferritin**

At pH levels below its pI (4.5, see reference 8), native ferritin has a positive charge, and it bound to the endothelial cell surfaces in the same manner as PCF. No binding was observed at pH levels above its pI.

**Neuraminidase**

Treatment with neuraminidase, followed by labeling with PCF at pH 1.8, resulted in a drastically diminished binding of PCF at the luminal endothelial surface (Figs. 10 and 11). At pH 3.5 and, perhaps, also at pH 5.5, the reduction in PCF binding after neuraminidase treatment was also discernible (Figs. 12 and 13). However, when
Figures 5-9 Polycationic ferritin treatment at pH levels ranging from 1.8 to 7.2. Fig. 5 shows the binding of PCF on the endothelial surface at pH 1.8, except at sites of LCV formation. The binding is essentially the same at pH 2.5 (Fig. 6). At pH 3.5 (Fig. 7), there is a moderate PCF binding at the sites of LCV formation. This, and also the PCF binding at the endothelial cell surface, increases at pH 5.5 and pH 7.2 (Figs. 8 and 9, respectively). × 54,000.
FIGURES 10-14 Polycationic ferritin binding at various pH levels after treatment with neuraminidase. Fig. 10 shows the control preparation, i.e., PCF after treatment at pH 1.8 in the same medium, for the same length of time, but without neuraminidase. After neuraminidase treatment, there is a drastic reduction of PCF binding at the endothelial surface at pH 1.8 (Fig. 11). There is no binding at the sites of LCV formation. At pH 3.5 (Fig. 12), there is somewhat more PCF binding at the endothelial surface after neuraminidase treatment than at pH 1.8. An occasional ferritin particle is seen at the sites of LCV formation. The PCF binding after neuraminidase increases at the endothelial cell surface as well as at the sites of LCV formation at pH 5.5 (Fig. 13) and pH 7.2 (Fig. 14). × 54,000.
Phosphotungstic Acid

The PTA-treated preparations showed either no staining or an irregular and spotty staining of the luminal endothelial cell membrane of sinusoidal vessels. This is in contrast to the luminal endothelial surfaces of other blood vessels, such as capillaries and arterioles, which were consistently stained with PTA. At the places of LCV formation in the sinusoidal endothelium, there was, however, always a distinct reaction with PTA (Fig. 15). In material that was fixed by immersion and then stained with PTA, no PTA staining of plasma components was found.

DISCUSSION

The positively charged CI sol used at low pH (1.8-2.0) is a dependable indicator of the presence of the carboxyls of membrane sialoglycoproteins and sialoglycolipids. At pH 1.8-2.0, the carboxyls of sialic acid, the pKa of which is estimated to be ~2.6 (49), are sufficiently dissociated for binding with the positive iron sol (5, 16, 51). Present results indicate that PCF is equally reliable for sialic acid at low pH, with the added benefit that it can also be used at higher pH levels than CI due to its stability. At the higher pH levels at which PCF can be used, acidic groups of higher pKa become available and, consequently, are stained with PCF as presently demonstrated.

The failure of CI and of PCF to bind at low pH to the endothelial cell membrane at sites of LCV formation appears to indicate that, at these endocytic sites, changes occur in the sialic acid moiety of glycoproteins and/or glycolipids of the cell membrane. Whether these changes are the result of the removal of sialic acid residues, the result of "masking" by nonsialated membrane components, or the result of a lateral displacement of this particular anionic membrane component remains unclear. Regarding the last alternative, a reversible aggregation of anionic sites on erythrocyte ghost membranes has been reported (30). A relatively rapid translocation of anionic sites over more substantial distances and induced by PCF has also been observed in vitro on the surfaces of baby hamster kidney cells (17).

Skutelsky and Danon (47) have examined in vitro at physiological pH the distribution of anionic sites of the luminal endothelial surfaces of excised nonsinusoidal blood vessels in the presence of PCF. They report a segregation of anionic sites into "clusters", as identified by aggregated PCF, followed by a detachment of these sites from the cell surface. In addition, they report the uptake of PCF by the endothelial cells followed by a transcellular transport and exocytosis at the abluminal endothelial surface. Of particular interest with respect to present findings is their observation that, after fixation with glutaraldehyde and staining with CI at pH 1.8, no anionic sites were found between the clusters induced by the PCF ligand in vitro. Taken together, these observations suggest the possible detachment of anionic groups or of entire glycoproteins from the cell membrane in the endocytic process at sites of LCV formation.

Certain previous studies have reported changes in ionic surface charge concomitant with the en-
endoctic process (6). Nagura et al. (29) found that a decrease in negative charge occurred during phagocytosis by macrophages and by ascites tumor cells. Skutelsky and Farquhar (48) reported a low degree of CI binding by the cell surfaces of macrophages, an observation that is confirmed in the present work (Fig. 3). Although present experiments at low pH levels indicate the apparent lack or masking of sialic acid at endocytic sites, the PCF binding experiments at higher pH levels indicate that, at physiological pH levels, there is yet an anionic surface at these sites. Whereas Noseworthy et al. (31) reported that removal of as much as 50% of the anion sialic acid does not affect the phagocytic function of polymorphonuclear leukocytes, Weiss et al. (50) found, on the basis of in vitro studies of monocytes, that neuraminidase treatment results in an increase in phagocytic uptake. In general, then, one might postulate that the presence of sialic acid at the cell surface is not essential for endocytosis. It is possible, in fact, that its presence is antagonistic to this process.

The suggestion of Quinton and Philpott (38) that anionic sites play a role in stabilizing membrane structures also deserves consideration here. These investigators found a marked loss of the membrane rigidity of epithelial cells after treatment with cationic polymers such as poly-L-lysine, protamine, and histone. Although the precise mechanism of this loss of membrane stability is, in view of the fluid membrane concept (46), not clear, it can be assumed that the formation of endocytic sites, such as is seen in LCV formation, involves a localized change in the normal fluidity of the cell membrane. Present experiments suggest that the exclusion of sialic acid residues, and presumably thereby of certain sialoglycoproteins and/or sialoglycolipids, at these sites may be of significance in the deformability of the surface membrane associated with endocytosis.

The pattern of PTA staining of the luminal surface of the sinusoidal endothelium also indicates a difference between the free surface and sites of LCV formation. The free surface of sinusoidal endothelial cells is largely PTA-negative, with only occasional sites showing a positive reaction. In contrast, endocytic sites were consistently PTA-positive. Unfortunately, the interpretation of this staining in chemical terms is difficult because of differences of opinion regarding the mechanisms of PTA binding to its organic substrate. Some investigators hold the view, based on the parallel occurrence of the periodic acid-Schiff reaction and PTA staining, that PTA is reasonably specific for glycosaminoglycans and glycoproteins (1, 14, 27, 32–34, 39–41). On the other hand, this supposition of binding to polysaccharides has been rejected by other investigators, arguing that PTA interacts with positively charged groups in general, and that because, at the low pH levels at which PTA staining is performed, many molecular species become cationic, no specificity for polysaccharides can be assumed (17, 35–37, 43–45). The demonstration that PTA does not react with sialic acid (37) is, however, in agreement with the present observation that the endothelial surfaces of the myeloid sinusoids, which contain sialic acid residues, also show a low degree of PTA staining. In this respect, the luminal surface membrane of the sinusoidal endothelium is clearly different from that of other vessels which do consistently react with PTA (39).

In summary, this study demonstrates that PCF is useful to indicate anionic sites at cell surfaces in fixed material; that PCF staining of cell surfaces at pH 1.8 is largely due to the sialic acid moieties of membrane glycoproteins and/or glycolipids; that the luminal sinusoidal endothelial cell plasma membrane contains evenly distributed sialoglycoproteins and/or sialoglycolipids except at sites involved in LCV formation: either the sites are devoid of sialoglycoproteins and/or sialoglycolipids, or their respective sialic acid moieties are blocked; and that these plasma membranes also contain evenly distributed anionic sites of a higher pKa than sialic acid, including those membrane areas involved in LCV formation. These sites most likely represent the carboxyl groups of amino acids.

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