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FERRITIN HYDRAZIDE, A NOVEL COVALENT ELECTRON DENSE REAGENT FOR THE ULTRASTRUCTURAL LOCALIZATION OF GLYCOCONJUGATES

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SUMMARY: Ferritin hydrazide was prepared by coupling horse spleen ferritin with an excess of bis-hydrazides, via carbodiimide activation of the protein carboxyl groups. The ferritin hydrazide was used for the one step direct covalent labeling and ultrastructural localization of periodate oxidized sialyl residues, or other aldehyde groups, on cell surfaces of erythrocytes and lymphocytes. In contrast to previous approaches, which have been based on electrostatic interactions, our method does not affect the cell surface charge. The method is also much simpler then the three-step affinity cytochemical techniques based on avidin-biotin interaction. To the best of our knowledge, this study represents the first example of covalent labeling of cell surfaces with an electron dense material.

INTRODUCTION

Cell surface oligosaccharides play an important role in cellular recognition processes and in cell-cell interactions (1). Methods enabling the analysis of the spatial and topographical distribution of the glycoconjugates on cell surfaces are highly required. Sialic acid, which occupies a terminal position in the main glycoconjugates, is one of the most prominent components of the mammalian cell surface. Therefore, several techniques have been developed for the determination and visualization of this sugar on the cell surface membrane. Among the techniques available for the labeling of sialoglycoconjugates are radioactive labeling with periodate-sodium borotritiate (2) and fluorescent labeling with rhodamine and fluoresceine hydrazides (3).

Ferritin has been introduced as a marker for electron microscopy because of its characteristic structured electron opacity (4). Conjugation of ferritin to biologically active molecules (such as antibodies, hormones and lectins), creates a complex between the electron dense material and the receptor

Abbreviations: FHZ - ferritin hydrazide; DDW - double distilled water; PBS - phosphate-buffered saline, pH 7.4; SCB - sodium cacodylate buffer pH 7.2.

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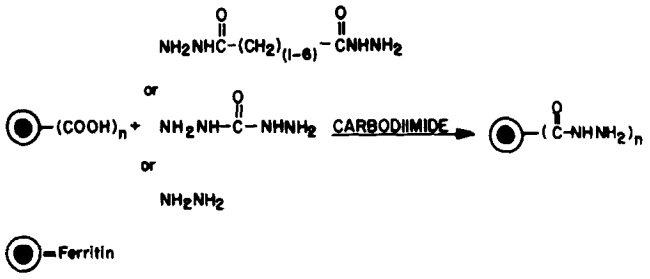

Fig 1. A general scheme for the synthesis of ferritin hydrazide.

specific molecule (5), the specificity of which resides in the biologically active molecule. Cationized ferritin is used as a reagent for the ultrastructural localization of membrane anionic sites including sialic acid (6). More recently, the specific ultrastructural localization of sialic acid has been achieved utilizing the ferritin - avidin - biotin system in a three step reaction (7,8).

In this communication we describe the synthesis of ferritin hydrazide (FHZ) and its use for the one step direct covalent labeling, and subsequent electron microscopic visualization, of periodate oxidized sialic acid or other aldehyde groups of cell membrane surfaces. Due to its simplicity we anticipate its rapid application as an ultrastructural probe of cell surfaces.

MATERIALS AND METHODS

Horse spleen ferritin (6X crystallized, cadmium removed) was obtained from Miles. The water soluble carbodiimide: 1-ethyl-3(3-dimethyl-amino propyl)-carbodiimide HCl, was purchased from Sigma. Sodium periodate from BDH. Adipic acid dihydrazide was synthesized (9). Sodium cacodylate , glutaraldehyde, osmium tetroxide and the embedding medium Poly/Bed 812, were obtained from Polysciences. Thymocytes from female BALB/c mice were isolated as previously described (10). Human erythrocytes were separated from heparinized, freshly donated blood.

Synthesis. FHZ was synthesized as described in Fig. 1. Adipic acid dihydrazide 40 mg was dissolved in 0.9 ml of double distilled water (DDW) with stirring. The pH was adjusted to 4.5 with 0.2 M HCl solution and 0.2 ml of a ferritin solution (57 mg/ml, determination based on the absorption at 440 nm) was added. The tube was gently shaken and 30 mg of the solid water soluble carbodiimide was added. The test-tube was again gently shaken and the reaction mixture was left at room temperature for 12 h. The mixture was then diluted to 12 ml with phosphate buffered saline (pH 7.4), and dialyzed twice at 4°C against 1L PBS for 12 h. The FHZ, now at a concentration of about 1 mg/ml, is ready for use.

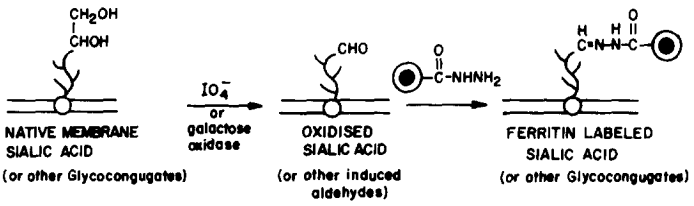

Fig. 2. Labeling procedure of intact cell membrane glycoconjugates by ferritin hydrazide.

Labeling of cells. The procedure for cell surface labeling with FHZ is shown schematically in Fig. 2. Human erythrocytes or mouse thymocytes (1 ml of PBS-washed cells $5 \cdot 10^7$ cells/ml) were put in an ice bucket and 0.5 ml of 3 mM $NaIO_4$ in PBS was added to give a final concentration of 1 mM sodium periodate. After 20 minutes at 0°C the cells were washed twice with PBS and resuspended in 0.5 ml PBS. The FHZ solution (0.5 ml) was added to the cell suspension to give a final concentration of 0.5 mg/ml FHZ and the reaction was allowed to proceed for 30 minutes at room temperature.

Fixation and embedding. After FHZ treatment the cells were washed twice with PBS, once with 0.09 M sodium cacodylate buffer pH 7.2 (SCB) and resuspended in 0.5 ml of SCB. Glutaraldehyde in SCB was added to give final concentration of 0.8%. After 30 minutes at room temperature the cells were washed twice with SCB, pelleted by centrifugation and post fixed for 1 h with 1% osmium tetroxide in SCB. The pellets were washed twice with DDW and then 0.5% uranyl acetate in DDW was added for 1 h. After removal of the uranyl acetate, the cells were dehydrated in ethanolic solutions (from 50% to 100%) and embedded in the Poly/Bed 812 medium, using conventional procedures (11).

Electron microscopy. Sections of approximately 500 Å-thick were cut with a diamond knife on a Sorvall ultramicrotome, mounted on naked 300-mesh copper grids and examined under the Philips-300 electron microscope. The sections were photographed at a magnification of 27,000 and accelerating voltage of 80 kV.

RESULTS

Synthesis and characterization of ferritin hydrazide. The FHZ was prepared by the direct coupling of the ferritin carboxyl groups with hydrazine, carbonyl hydrazide or other bis-hydrazides (usually adipic acid dihydrazide), in the presence of water soluble carbodiimides (Fig. 1). The reaction was performed at the pH range 4-5, and was efficient over the wide range of reagents tested. For our localization studies we preferred the FHZ with adipic dihydrazide because of its longer spacer arm. The FHZ obtained is polyvalent since ferritin contains about 550 carboxyl groups, many of which reacted with the hydrazides. The number of hydrazide groups per ferritin has not yet been determined. The polyvalency of the FHZ was also demonstrated by its ability

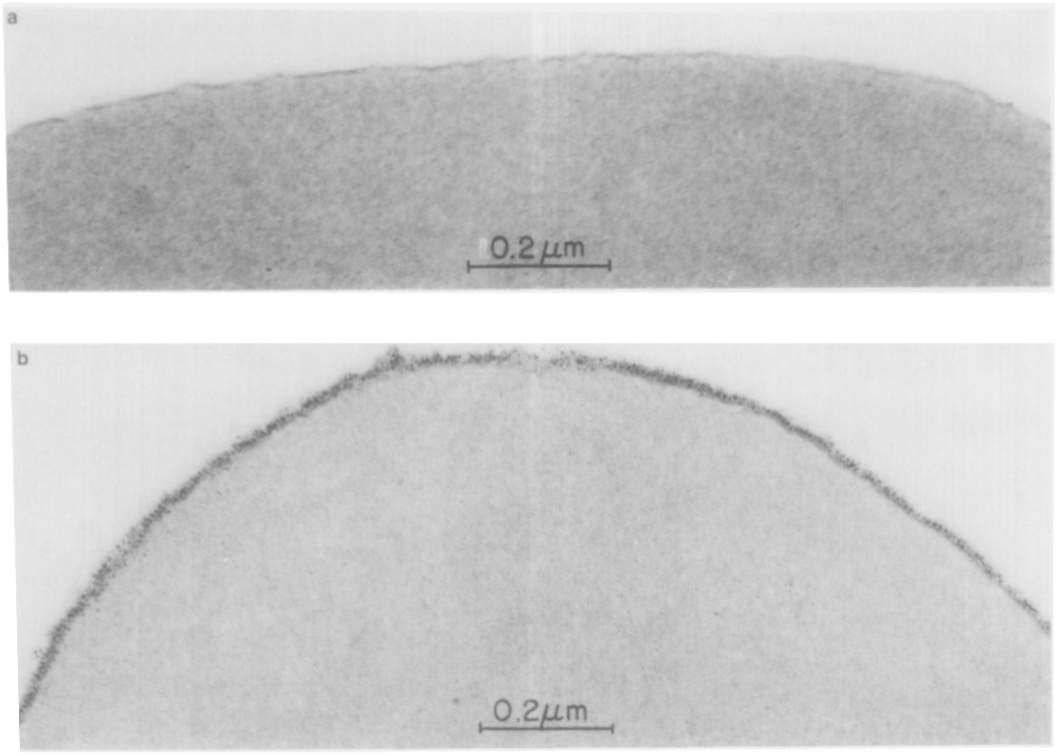

Fig. 3. Transmission electron micrographs of human erythrocyte surfaces treated with 0.5 mg/ml ferritin hydrazide solution (a) before and (b) after periodate oxidation.

to induce cap formation on lymphocytes (data not shown). The FHZ retains the spherical electron-dense nature of the ferritin iron core. The large dimensions of FHZ make it an exclusive reagent for the outer cell surface, when applied to intact cells.

Labeling and localization of sialic acids on cell surfaces. The steps involved in labeling of sialyl residues with FHZ are schematically shown in Fig. 2. No binding of FHZ was evident on human red blood cells (Fig 3a) or on mouse lymphocytes (Fig. 4a), without previous periodate oxidation. In contrast, numerous ferritin particles uniformly distributed were observed on the human erythrocytes and on the mouse lymphocytes after periodate oxidation (Figs. 3b and 4b, respectively). In both kinds of cells, the attached ferritin particles were seen on perpendicularly sectioned membranes, near the outer

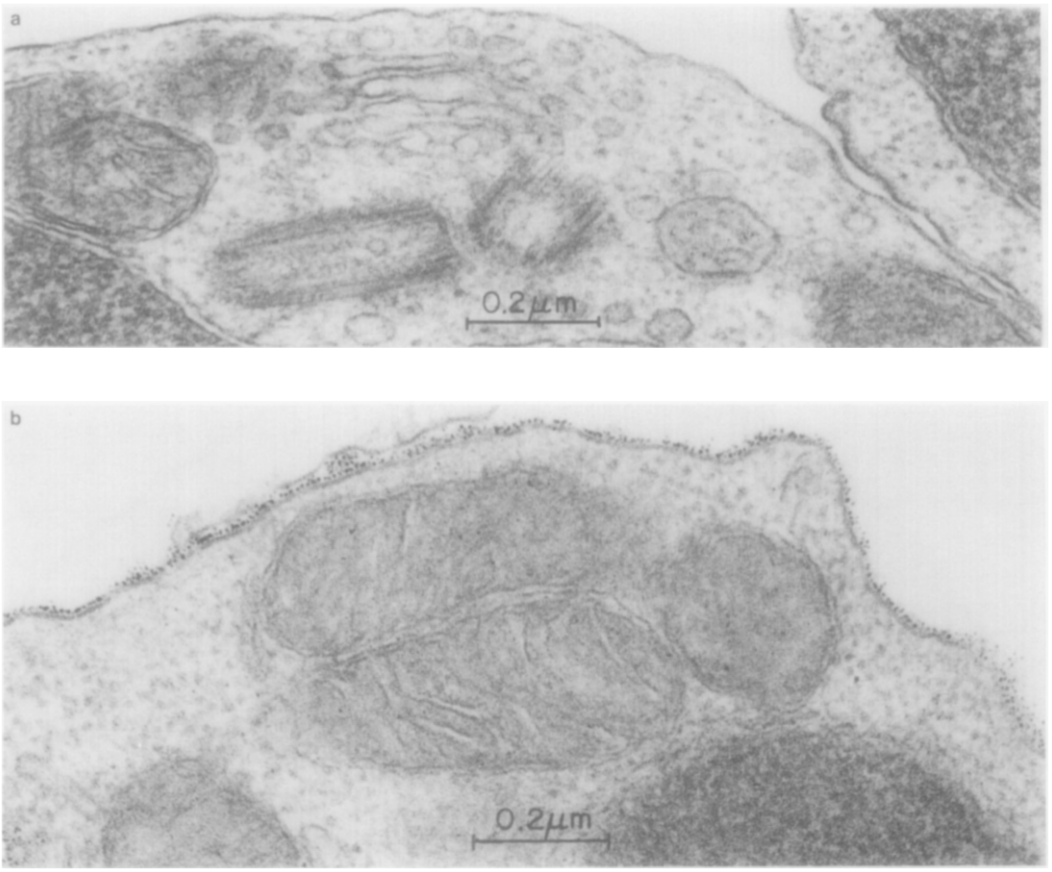

Fig. 4. Transmission electron micrographs of mouse thymocyte surfaces treated with 0.5 mg/ml ferritin hydrazide solution (a) before and (b) after periodate oxidation.

dense line of the cell membrane. The average distance which separates the ferritin iron core from the outer dense line of the membrane was less than that observed with avidin-biotin complex (7) and greater than with cationized ferritin (6). The non aggregative nature of FHZ is also shown by Figs. 3b and 4b. In addition, no damage to the membrane or lysis of the cells were observed upon reaction of FHZ with the cell membrane.

DISCUSSION

The intensive study of the role of glycoproteins and glycolipids in biological membranes,calls for the development of new highly specific

reagents which can chemically label these molecules. The highest order of resolution, concerning the structural and functional role of these determinants in the architecture of the cell surface, is achieved by specific cytochemical techniques and electron microscopy. Two methods were recently introduced using ferritin as an electron-dense reagent for the labeling of sialic acid. One involves cationized ferritin (6), the other is based on the high affinity constant of the interaction between the ferritin-avidin conjugate and biotin covalently grafted to the cell surface (8). The first method, based on electrostatic interactions, is not only confined to sialic acid, but is also sensitive to other anionic sites.

The method described in this publication is nearer in character to the ferritin-avidin-biotin method, since the first reaction, the periodate oxidation of the cell surface sialic acid, is the same in both labeling procedures. However, our new approach is much simpler since the second step, the covalent coupling with FHZ results in the direct visualization of the sialic acid residues, while in the previous procedure the cells had to be reacted sequentially with biotin hydrazide and ferritin-avidin. The preparation of ferritin-avidin conjugate represents a major problem since it can result in high molecular weight aggregates, loss of biological activity, and tends to precipitate on storage. On the other hand, FHZ is easy to prepare, does not precipitate on storage, and the labeling consists of a single layer of ferritin molecules with no tendency to aggregate.

The electron dense FHZ is recommended as the reagent of choice for the ultrastructural localization and labeling of cell surface sialic acid and other aldehyde groups for the following reasons: a) It is highly specific for aldehyde groups (e.g. periodate oxidized sialic acid, galactose oxidase oxidized galactose) and there is no need to reduce the Schiff-base formed after the reaction due to its polyvalency; b) It is used at physiological conditions and no prefixation of the cells is required; c) It does not alter

the charge of the sialic acid, since the reaction is performed on site distinct from the C-1 carboxyl group.

FHZ is currently being used in our laboratory to study (by transmission electron microscopy, scanning electron microscopy, and freeze-fracture techniques) the influence of different metabolites on both vertical and horizontal architecture of some intact cell membrane glycoconjugates.

FHZ, together with the fluorescent hydrazides recently introduced (3), are useful tools for the localization and visualization of cell-surface events connected with glycoconjugates.

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