THE LOCALIZATION OF SPECTRIN ON THE INNER SURFACE OF HUMAN RED BLOOD CELL MEMBRANES BY FERRITIN-CONJUGATED ANTIBODIES

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ABSTRACT

Spectrin, a major protein constituent of mammalian red blood cell membrane preparations, has been localized on the inner surface of human red blood cell membranes by techniques that utilized specific ferritin-conjugated antibodies and fixation of membranes shortly after hemolysis so as to allow penetration of the ferritin-antibody labels. The labeling of spectrin was shown to be specific by the following criteria. (*a*) Nonhomologous ferritin-conjugated antibodies did not specifically bind to either membrane surface. (*b*) Blocking the membrane-bound spectrin with excess unconjugated antispectrin antibodies prevented ferritin-antibody labeling. (*c*) Removal of spectrin by treating the membrane preparation with a low ionic strength buffer containing ethylenediaminetetraacetate and β-mercaptoethanol prevented labeling by specific ferritin-conjugated antibodies.

INTRODUCTION

It has been recently shown that red blood cell membrane preparations from a variety of species (rabbit, horse, sheep, guinea pig, and human) contain a fibrous protein called spectrin which constitutes approximately 20% of the total membrane protein (3, 5, 6, 11). Spectrin extracted from preparations of human red cell membranes in low ionic strength buffers containing ethylenediaminetetraacetate (EDTA) and β-mercaptoethanol has been purified and shown to be free of lipid and carbohydrate and is composed of aggregating protein monomers of approximately 140,000 mol wt (3).

The location of spectrin in red cell membranes has not been firmly established heretofore; however, examination of the morphology of membranes in the electron microscope indicates that filamentous structures are normally present on what appears to be the inner surface of the membrane after hypotonic lysis (4). The same structures cannot be identified after dialysis of the membranes against low osmolality buffers containing EDTA and β-mercaptoethanol, a treatment known to dissociate spectrin from the membranes. Other indirect evidence to support the suggestion that spectrin is localized on the inner membrane surface is that antibodies to purified spectrin do not appear to bind to intact red blood cells since the cells are not agglutinated (6).

The present study was initiated for the purpose

of determining directly the location of spectrin with specific ferritin-conjugated antibodies (9), using techniques developed to allow penetration of the ferritin-antibodies into red cell ghosts by fixing transient holes in the membrane (8).

MATERIALS AND METHODS

Horse spleen ferritin (six times recrystallized, cadmium free) was obtained from Miles Laboratories, Inc., Elkhart, Ind., and was further purified by cadmium sulfate crystallization and precipitation in 50% ammonium sulfate. The ferritin was then ultracentrifuged so as to remove traces of apoferritin, and finally the ferritin solution was passed through a 0.05 μ Millipore filter (Millipore Corp., Bedford, Mass.) and stored under sterile conditions. Toluene-2,4-diisocyanate, obtained from Polysciences, Inc. Rydal, Pa., was stored at −10°C and melted at room temperature immediately before use. Glutaraldehyde was freshly prepared by distillation of 50% glutaraldehyde obtained from Union Carbide Corp., New York.

Influenza virus (strain PR 8) was grown in eggs and absorbed and eluted from red cells, followed by ammonium sulfate precipitation and centrifugation in sodium tartrate gradients. After dialysis and concentration, the influenza virus titer was approximately 10,000 hemagglutination units/ml (2).

Antisera and Ferritin-Antibody Conjugates

The γ-globulin fraction of rabbit anti(tobacco) fraction I protein, a generous gift of Drs. N. Kawashima and S. G. Wildman, was separated from antisera by ammonium sulfate precipitation and further purified by diethylaminoethyl (DEAE) cellulose chromatography. It was available for use as a control ferritin conjugate of a nonhomologous rabbit γ-globulin. Rabbit anti(human) spectrin was obtained by immunizing rabbits with purified human spectrin in complete Freund's adjuvant. Double diffusion with the antispectrin antiserum against purified human spectrin yielded a single precipitin band (5). Before fractionation with ammonium sulfate, the antiserum was absorbed with fresh human red cells (O+) overnight at 5°C.

The specific γ-globulin fractions were conjugated to ferritin using toluene-2,4-diisocyanate by previously published methods (7, 9). The ferritin-conjugated antibodies (together with ferritin-conjugated normal γ-globulin) were separated from ferritin and unconjugated γ-globulin by chromatography on a 1 × 40 cm column of (Bio-Rad Laboratories, Richmond, Calif.) A 1.5 M agarose (200–400 mesh). The first peak containing ferritin-conjugated antibody (Fig. 6) was concentrated and redialyzed against 0.05 M sodium phosphate, pH 7.5, and finally filtered through a 0.22 μ Millipore filter before storage in a sterile vial.

Red Blood Cell Ghosts

A sample of fresh human blood (B+) was collected in ACD (15 ml of 0.8% citric acid, 2.2% sodium citrate, 2.45% dextrose/100 ml of blood) and washed three times in 0.9% NaCl. The white cell coat was aspirated after each centrifugation. The cells were hemolyzed by the method of Dodge et al. (1) in 20 milliosmolar (mosmolar) sodium phosphate buffer, pH 7.4 (20PB), at 4°C by slowly adding 1 vol of washed cells (50% in 0.9% NaCl) to 10 vol of 20 PB with rapid stirring. The lysed cells were immediately centrifuged at 15,000 *g* for 15 min and resuspended in 310 mosmolar sodium phosphate buffer, pH 7.4 (310 PB). The pink membrane suspension was allowed to reseal for 30 min at 5°C and was washed twice in 310 PB.

Spectrin-free membranes were prepared by lysing washed cells in 5 mM Tris-HCl, 5 mM EDTA, 1 mM β-mercaptoethanol, pH 7.4 (Tris-EDTA). 1 vol of washed cells (50% in 0.9% NaCl) was added slowly to 50 vol Tris-EDTA,and the suspension was stirred for 30 min at 5°C. After centrifugation and resuspension six times alternately in Tris-EDTA and 310 PB containing 5 mM EDTA, the EDTA-treated membranes were suspended in 310 PB.

The washed membranes were then lysed and rapidly fixed a short time after lysis by a modification of the techniques used by Seeman (8) to fix transient holes in the membranes in the "open" position. To a 0.4 ml sample of the membrane pellet in 310 PB at room temperature was added 10 ml of 20 PB from a blow-out pipet, and 15–20 sec later 1 ml of a neutral 1 or 2% glutaraldehyde solution. The membranes were fixed for 60 sec, and the fixation was terminated by addition of 1 ml of 1 M ammonium carbonate. After a few minutes, the suspension was centrifuged and resuspended three times in 310 PB.

Ferritin-Antibody Labeling

Ferritin-antibody labeling was performed on normal and EDTA-treated membranes as follows. A 0.1 ml sample of the glutaraldehyde-fixed membrane pellet was incubated with the appropriate purified ferritin-conjugated antibodies at a conjugate concentration of 1–2 mg/ml for 20 min at room temperature. The membranes were freed of unbound conjugate by centrifugation and resuspension four times in 0.05 M sodium phosphate, pH 7.5. Blocking experiments were performed by first incubating the membranes with unconjugated anti(human) spectrin γ-globulin (20 mg/ml) for 30 min at room temperature and then

washing two times before labeling with ferritin-conjugated antibodies. Before further fixation, the outer surface of the membrane in some of the preparations was tagged with virus by suspending the membranes in 0.4 ml of a preparation containing 10,000 hemagglutination units/ml of purified influenza virus for 10 min at 0°C. The unbound influenza virus was removed by centrifugation of the red cell membranes in 310 PB.

Electron Microscopy

Ferritin-antibody–labeled membranes and controls were fixed in 2% OsO_4 in 0.1 M sodium phosphate buffer pH 7.4, for 2 hr at 4°C. After removing the excess osmium by centrifugation and resuspension in fresh buffer, the ghosts were dehydrated in ethanol and embedded in Epon 812. Silver-to-gold colored sections were mounted on uncoated grids and stained 5–10 min with 2% uranyl acetate. Lead staining was not used in order to enhance the ferritin contrast. The sections were examined in a Philips model 300 electron microscope.

RESULTS AND DISCUSSION

If isolated red blood cell membranes are placed into isotonic neutral buffers, they "reseal"; that is, they reform biconcave disc-shaped corpuscles which are impermeable to proteins. In this state, an antigen on the inner surface of the membranes would, of course, be inaccessible to specific ferritin-conjugated antibodies. Such membranes can, however, be made permanently permeable to proteins, if, as was shown by Seeman (8), they are once again placed in hypotonic media and then rapidly fixed in dilute glutaraldehyde. In our experiments, fixation with 0.1–0.2% glutaraldehyde 15–20 sec after making the cells hypotonic leaves 30–40% of the membranes in the permanently permeable, or "open," state when they are returned to isotonic medium. This was demonstrated by suspending the membranes in a nonspecific ferritin-conjugated γ-globulin and then, without extensive washing, embedding the cells.

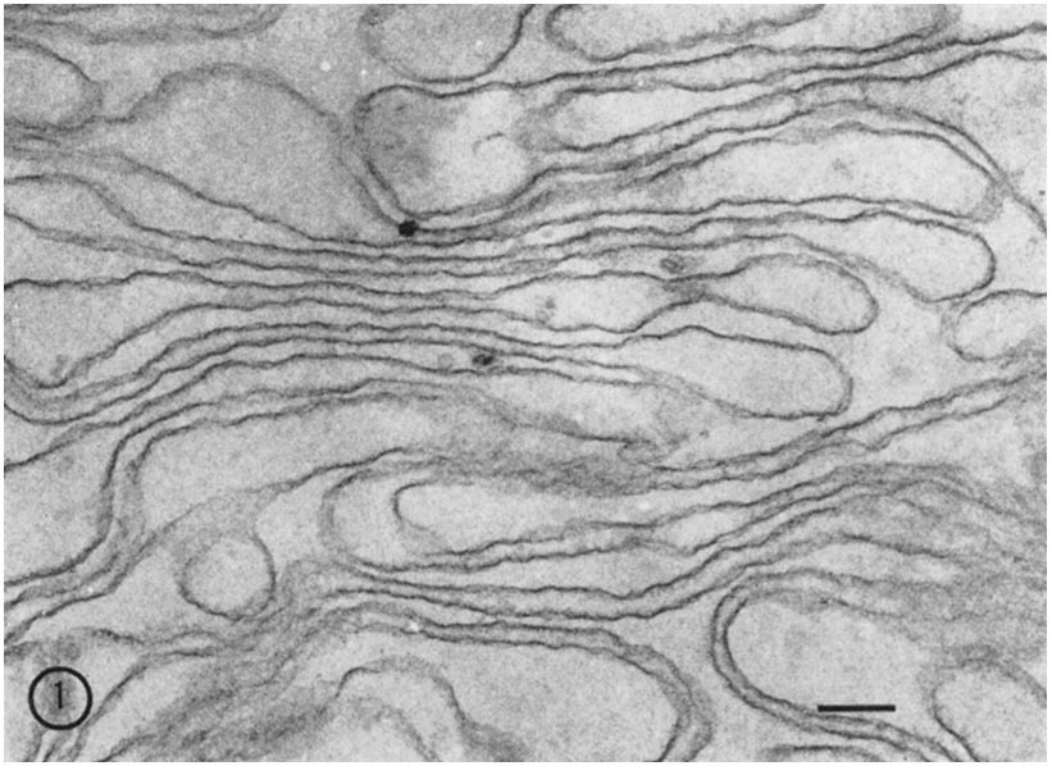

FIGURE 1 Human red blood cell ghosts fixed after hypotonic lysis and incubated with ferritin-conjugated anti(tobacco) fraction 1 protein. Washing removes the nonhomologous ferritin antibodies. Bar equals 0.2 μm.

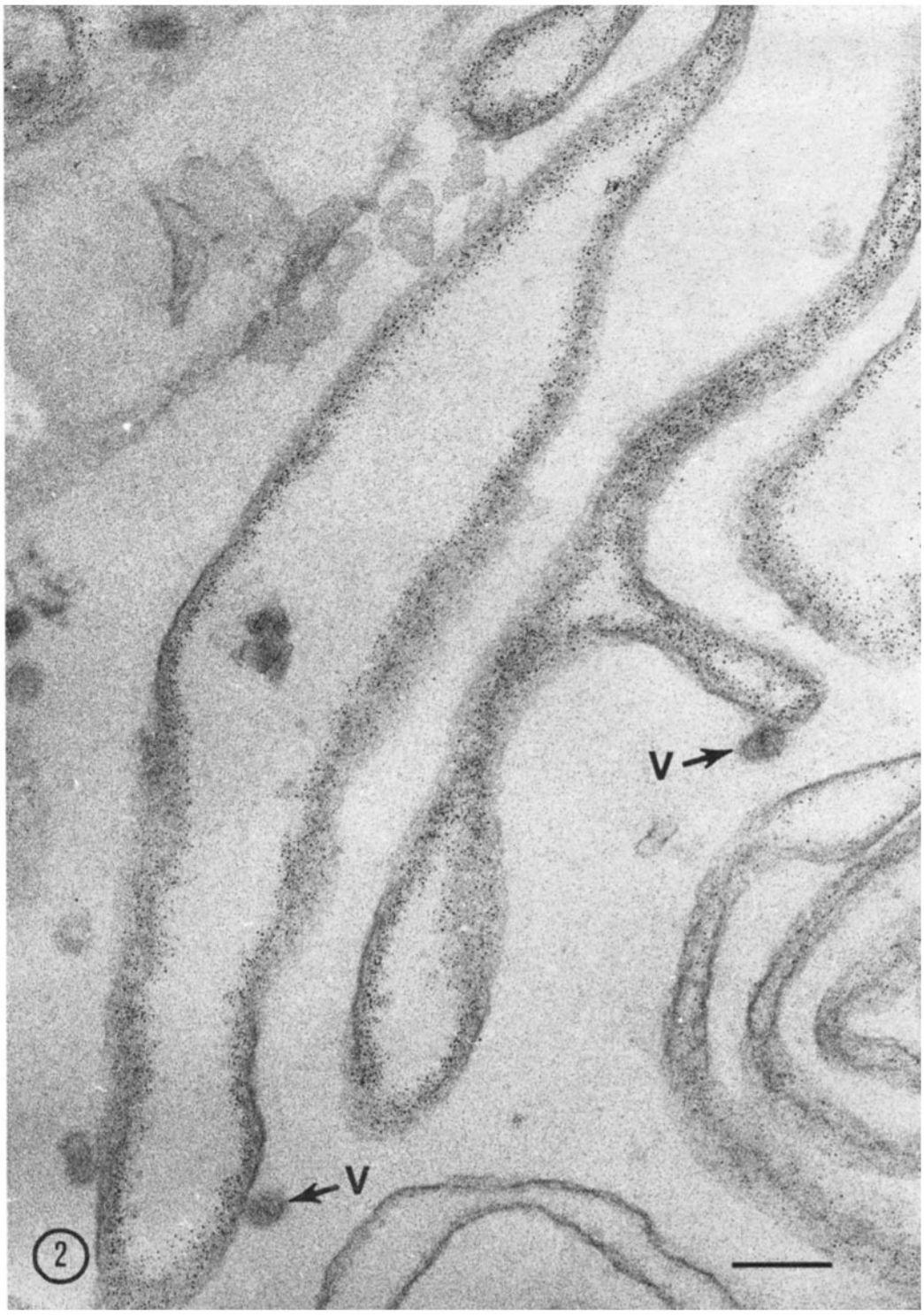

FIGURE 2 Human red blood cell ghosts fixed after hypotonic lysis and incubated with ferritin-conjugated anti(human) spectrin. After washing, ferritin antibodies are localized on the inner surface of the membranes. Influenza virus (*V*) was used to identify the outer membrane surface. Bar equals 0.2 μm.

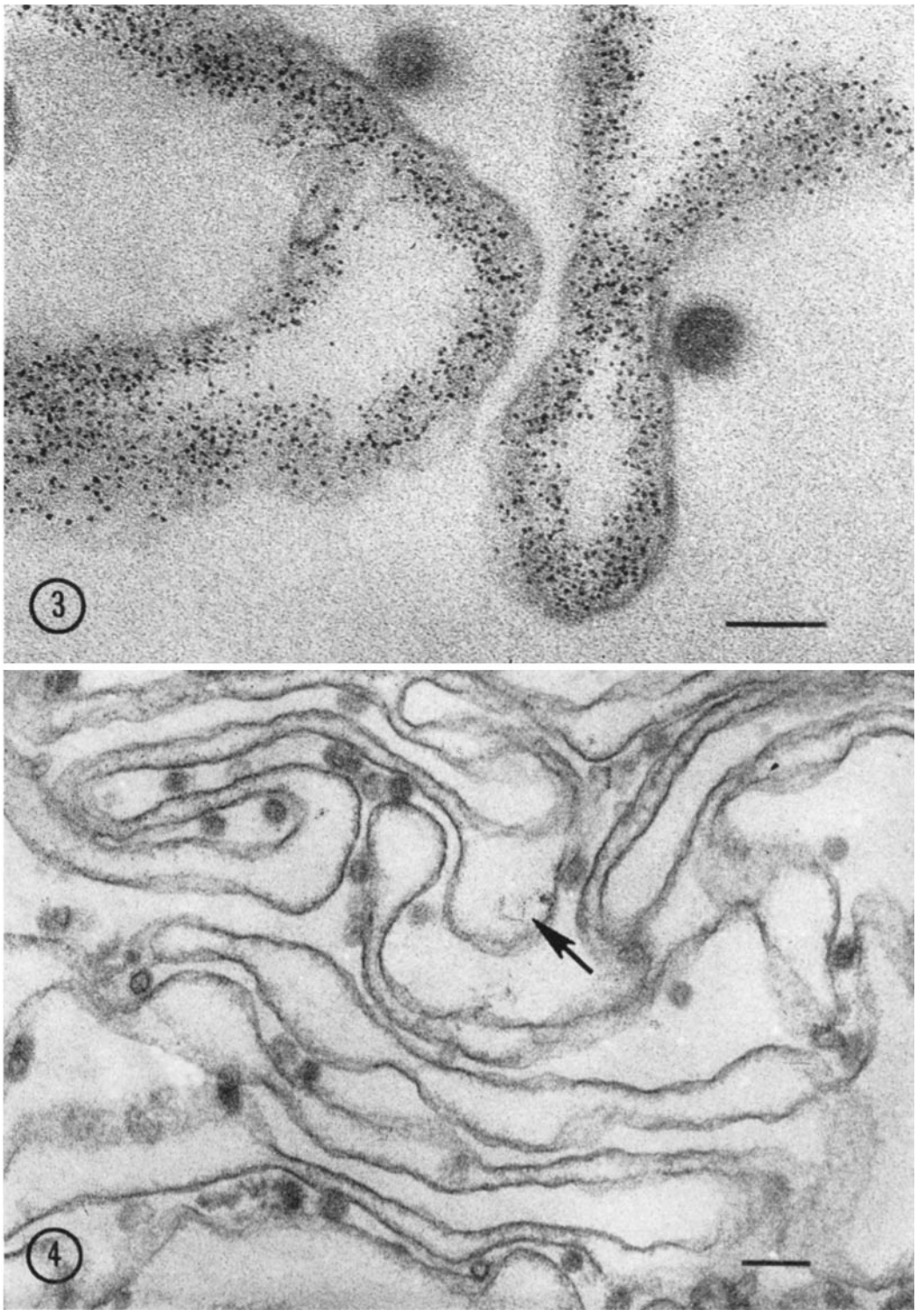

FIGURE 3 Legend is the same as Fig. 2. Bar equals 0.1 μm.

FIGURE 4 A blocking experiment. Legend is the same as Fig. 2 except that the red cell ghosts were incubated with unconjugated anti(human) spectrin before labeling with ferritin-conjugated anti(human) spectrin. A few ferritin antibodies are visible (arrow). Bar equals 0.2 μm.

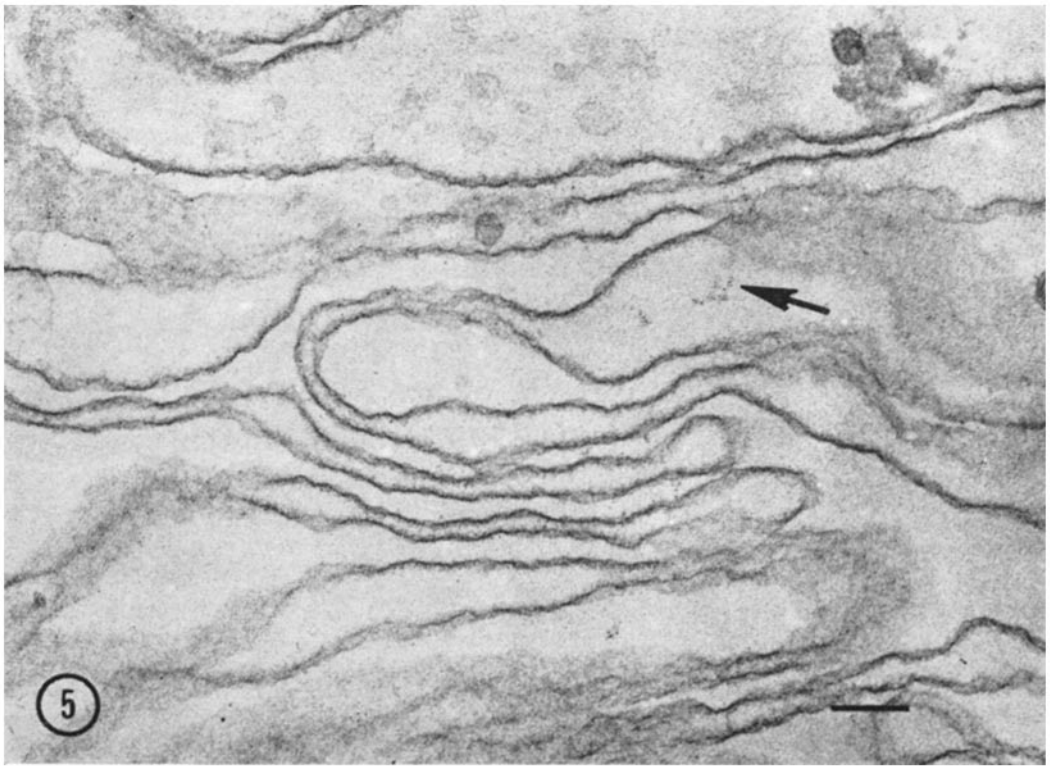

FIGURE 5 Human red blood cell ghosts were treated with a buffer containing EDTA-mercaptoethanol in order to remove spectrin before labeling with ferritin-conjugated anti(human) spectrin. Some spectrin remained (arrow) after EDTA-mercaptoethanol treatment, but the staining of ferritin antibody is quite low. Bar equals 0.2 μm.

In thin sections, the nonspecific ferritin was observed inside the open membranes. On the other hand, if such membranes were thoroughly washed by centrifugation four times before embedding, the nonspecific ferritin-conjugated γ-globulin was completely removed from inside the membranes (Fig. 1).

Red blood cell membranes fixed in the open state and treated with ferritin-conjugated antispectrin antibodies showed intense binding of ferritin exclusively localized to the inner surfaces of the membranes (Figs. 2 and 3). The outer surfaces of the membranes were marked by the influenza virus particles attached to the membrane virus receptors, as it has been shown (G. L. Nicolson and S. J. Singer, unpublished results) that influenza virus binds exclusively to the outer membrane surface. That the binding of the ferritin-conjugated antispectrin antibodies was specific was further demonstrated by the facts that (*a*) an initial treatment of the open membranes with unconjugated antispectrin antibodies blocked the subsequent binding of the specific ferritin-conjugates (Fig. 4); and (*b*) that spectrin-depleted membranes, prepared by EDTA-mercaptoethanol treatment, showed little binding of the ferritin-conjugated antispectrin antibodies (Fig. 5).

The patterns of specific ferritin-antibody staining observed in Figs. 2 and 3 indicate that after the various treatments involved in the preparation of the specimens, the spectrin is attached to the inner surface of the membranes, probably in a loose filamentous form extending several hundreds of Ångstroms from the surface, as had earlier been suggested (6).

The exclusive association of spectrin with the inner surface is yet another reflection of the chemical and structural asymmetry of the red

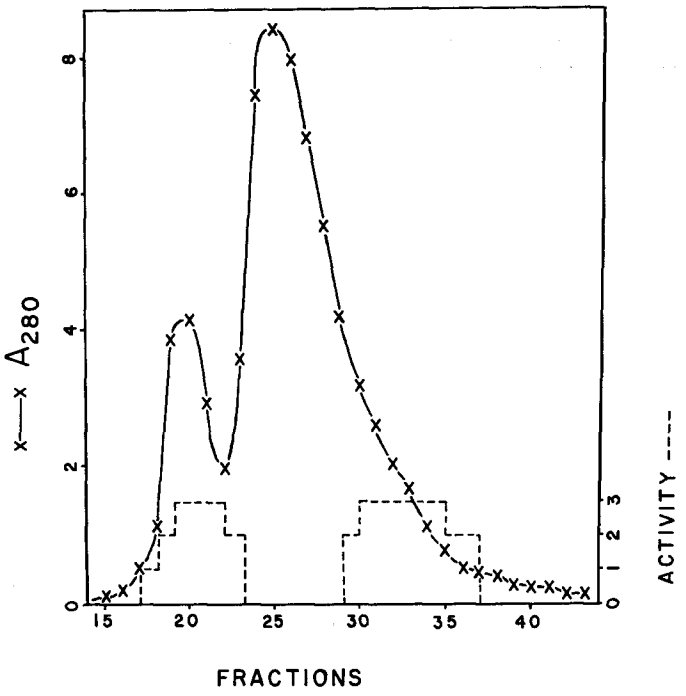

FIGURE 6 Chromatography of the solution of ferritin-antispectrin conjugates. A 0.5 ml sample was applied to a column of agarose A 1.5 m and eluted with 0.05 M sodium phosphate buffer, pH 7.5. Fractions (0.5 ml) were collected and measured for absorbance at 280 nm and for antispectrin activity (dashed lines) by double diffusion analyses against purified human spectrin. The activity was recorded qualitatively on a scale of 0–3 according to the density of the precipitin band.

blood cell membrane (see Marchesi and Palade, 4).

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