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Structural Alterations of the Human Erythrocyte Membrane Upon Influenza Virus Attachment

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Molecular events on the human erythrocyte membrane subsequent to influence virus binding were investigated by electron spin resonance (ESR) measurements after spin labeling of the cell membrane at different positions. Virus binding affected the glycocalyx structure as well as the physical state of the cytoskeleton at the inner leaflet, but not the lipid phase. A lateral reorganization of spin-labeled glycophorin was not indicated after virus attachment.

INTRODUCTION

After attachment of enveloped viruses to the cell, the viral membrane fuses directly with the plasma membrane, releasing the nucleocapsid into the cytoplasm (paramyxoviruses), or the virus particles are endocytosed at distinct microdomains of the cell surface—coated pits—forming coated vesicles (orthomyxoviruses [e.g. influenza virus], togavirus, rhabdovirus) (White *et al.*, 1983; Marsh *et al.*, 1982). The molecular events on the plasma membrane as well as on its surfaces immediately after virus binding are not well understood. To elucidate the molecular mechanism of virus entry, mammalian and avian erythrocytes were often used as a model system for target

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cells. Besides electron microscope studies, ESR measurements on the physical properties of the lipid phase after virus binding were performed (Bächi *et al.*, 1983; Lyles and Landsberger, 1976, 1977, 1978, 1979; Maeda *et al.*, 1975; Maeda *et al.*, 1981; Kuroda *et al.*, 1980). Only in the case of avian erythrocytes was an increased lipid fluidity detected (Lyles and Landsberger, 1976, 1978). It was assumed that a lateral redistribution of receptor molecules within the plane of the membrane influences the lipid phase, possibly triggered by a microtubule-like system. An involvement of the spectrin–actin meshwork in structural alterations of the plasma membrane was also stated by other groups (Bächi *et al.*, 1983; Sekiguchi and Asano, 1978; Loyter and Lalazar, 1980).

In the present study we investigated structural alterations of the human erythrocyte membrane subsequent to influenza virus binding by ESR in an extended way. The cell membrane was labeled at different positions. The surface coat (glycocalyx) was characterized by spin labeling in two different ways: (I) A positively charged spin probe with a long acyl chain was incorporated into the outer leaflet of the erythrocyte membrane. The N–O group of this probe exposed out of the bilayer is sensitive to events on the membrane surface (Laßmann and Herrmann, 1984; Herrmann *et al.*, 1985; Coughlin *et al.*, 1983; Hubbell *et al.*, 1970). (II) Sialic acid residues of glycophorin and glycolipids which are the attachment sites for the influenza virus were covalently spin labeled (Feix and Butterfield, 1980; Alpin *et al.*, 1979; Lee and Grant, 1979; Sharom and Grant, 1977). To investigate whether virus binding on the outer membrane surface would be manifested by changes in the physical state of the opposite side of the membrane, we labeled membrane proteins covalently by the protein-specific spin label MAL-6 which is mainly bound on the spectrin–actin meshwork (Barber *et al.*, 1985; Fung and Simpson, 1979; Butterfield *et al.*, 1976). Furthermore, lipid fluidity was explored by fatty acid spin labels. To distinguish between virus adsorption, virus-induced hemolysis and virus–cell fusion, experiments were performed at pH 7.4 (only adsorption) and at pH 5.2 (adsorption, hemolysis, and fusion) (Maeda *et al.*, 1975; Maeda *et al.*, 1981; Maeda and Ohnishi, 1980; Maeda *et al.*, 1981).

MATERIALS AND METHODS

Human erythrocytes of several donors were used (blood-bank Berlin–Lichtenberg). All experiments were performed not later than 48 hr after blood sampling (ACD–storage medium, 4°C). After removal of buffy coat and plasma (at 500*g*, 5 min) three subsequent washings in 5 vol. of phosphate buffered (5.8 m*M*, pH 7.4) or acetate buffered (20 m*M*, pH 5.2) physiological saline (150 m*M* NaCl) at 2,000*g* for 10 min followed. Erythrocyte ghosts were prepared according to the procedure of Dodge *et al.* (1963). If not stated otherwise, erythrocytes as well as ghosts were suspended and incubated in one of the media mentioned above.

Spin labeling of erythrocytes and ghosts with the fatty acid spin labels I(10,3) (2-(3-carboxypropyl)-2-decyl-4,4-dimethyl-3-oxazolidinyloxyl, Reanal, Budapest) and I(1,14) (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl, Synvar, Palo Alto) and of erythrocytes with the cationic spin probe CAT 16 (1-oxyl-2,2,6,6-tetramethyl-4-dimethyl aminopiperidine-cetyl bromide, Inst. Org. Chemistry,

Bulgarian Acad. Sci., Sofia) were performed as already described (Laßmann and Herrmann, 1984; Herrmann *et al.*, 1985). Sialic acid residues of glycoproteins as well as glycolipids of the surface coat of erythrocyte ghost membranes were covalently labeled with TEMPAMINE (2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl, Reanal Budapest) overnight (4°C) following the procedure of Feix and Butterfield (1980) (Herrmann *et al.*, 1985). Labeling procedure was performed in phosphate buffered (5.8 m*M*) saline (150 m*M* NaCl) at pH 8.0. SH-groups of proteins of ghost membranes were labeled with the protein-specific label MAL-6 (2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl, Reanal Budapest): 40 μg of the label was added per mg ghost protein and incubated overnight at 4°C (pH 7.4). In all cases excess of spin label was removed by repeated washings.

After spin labeling, virus treatment was performed. Erythrocytes were resuspended to a final hematocrit of 2.5% at 4°C (pH 7.4 or 5.2). The number of ghosts per volume corresponded to that of intact cells at a hematocrit of 2.5%. After addition of different amounts of the influenza virus A_0PR8 (2.3×10^4 HAU/mg virus protein) which was kindly supplied by the Institut für Angewandte Virologie, Berlin–Schöneweide, an incubation at 4°C for 10 min followed.

For ESR measurements pellets were put into a flat quartz cell for aqueous solutions. ESR spectra were recorded on a spectrometer ERS 231 (Acad. Sci., GDR) with a variable temperature equipment. The temperature was measured by a small thermistor inserted into the sample cell.

The order parameter S of the membrane spectra I(10,3) was determined according to the method of Griffith and Jost (1976). In case of I(1,14) an apparent correlation time τ_B given below was calculated. For the spin label CAT 16 the external peak distance $2A_Z'$ was used in the low-temperature region (5°C) as a relative measure of mobility of the probe (Laßmann and Herrmann, 1984; Herrmann *et al.*, 1985). At 37°C with fast motion of the label the apparent correlation time τ_{CAT} was derived from the spectra (Laßmann and Herrmann, 1984; Herrmann *et al.*, 1985)

$$\tau_{CAT}\{ns\} = 6.65 \times 10^{-10} \times \Delta H_{+1}\{G\} \times ([I_{+1}/I_{-1}]^{1/2} - 1)$$

For comparison (Feix and Butterfield, 1980; Butterfield *et al.*, 1983) the motion of spin labeled sialic acid residues was described by the following correlation times (Butler and Smith, 1978):

$$\tau_B\{ns\} = -5.9 \times 10^{-10} \times \Delta H_0\{G\} \times ([I_0/I_{+1}]^{1/2} - [I_0/I_{-1}]^{1/2})$$

$$\tau_C\{ns\} = 6.55 \times 10^{-10} \times \Delta H_0\{G\} \times ([I_0/I_{+1}]^{1/2} + [I_0/I_{-1}]^{1/2} - 2)$$

with $\Delta H_{+1}, \Delta H_0, I_{+1}, I_0, I_{-1}$ the linewidths and the amplitudes of the low field, midfield and high field lines.

RESULTS

Lipid Phase Fluidity

The membrane spectrum of the fatty acid spin label I(10,3) reflects the properties of the lipid phase near the head group, whereas that of I(1,14) the hydrophobic part near

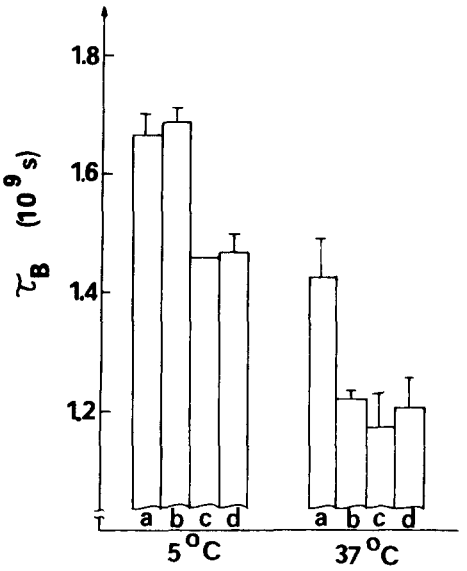

Fig. 1. The apparent correlation time τ_B of I(1,14) incorporated into the human erythrocyte membrane (150 mM NaCl, pH 5.2). a-control; b,c-after incubation of erythrocytes at 4 °C for 10 min in the presence of influenza virus (40 μg virus protein/ml) (b) and, subsequently, for 30 min at 37 °C in the presence of influenza virus (c); d-erythrocyte ghosts prepared by incubation in hypotonic media (pH 5.2) for 30 min at 37°C. The standard error of estimate is presented.

the center of the bilayer. At pH 7.4 the order parameter S of I(10.3) as well as the apparent correlation time τ_B of I(1,14) were not affected by virus attachment between 5°C and 37°C (spectra were recorded at various temperatures: 5, 15, 25, and 37°C). This was also established in media of pH 5.2 at temperatures lower than 37°C. After heating the cells to 37°C a significant decrease of τ_B (Fig. 1) and S (not shown) was found at pH 5.2 in the presence of influenza virus (40 μg virus–protein/ml). This was accompanied by an intensive hemolysis and cell–cell fusion as checked by phase contrast microscopy. The effect on τ_B and S was not virus specific since the same alteration of these parameters were found after hemolyzing erythrocytes in a hypotonic medium (pH 5.2) at 37°C and resuspending in physiological saline (pH 5.2) (Fig. 1). Furthermore, no effect on the spectra of I(10,3) and I(1,14) of ghost membranes was detected at pH 5.2 (37°C) after virus addition.

Influence of Virus Binding on the Membrane Surface Coat

Typical spectra of CAT 16 incorporated into the erythrocyte membrane are shown in Fig. 2a. The line shapes of the spectra of this label which is sensitive to alterations directly on the outer membrane surface have already been discussed (Laßmann and Herrmann, 1984; Herrmann *et al.*, 1985; Coughlin *et al.*, 1983; Hubbell *et al.*, 1970). We investigated the motional behavior of CAT 16 at pH 7.4 and 5.2 in the presence of influenza virus (40 μg virus protein/ml). The significant increase (t-test, $\alpha = 0.05$) of the external peak distance $2A_Z'$ (5°C) as well as of the correlation time τ_{CAT} (37°C) at both pHs in comparison with the control suggested a hindered motion after virus attachment (Fig. 3). No significant pH dependent differences were observed. The half-line width of the low field line at 5°C remained constant within the experimental error.

A spectrum of covalently labeled sialic acid residues of the glycocalyx of erythrocyte membranes is presented in Fig. 2b. Under our conditions about 70% of the

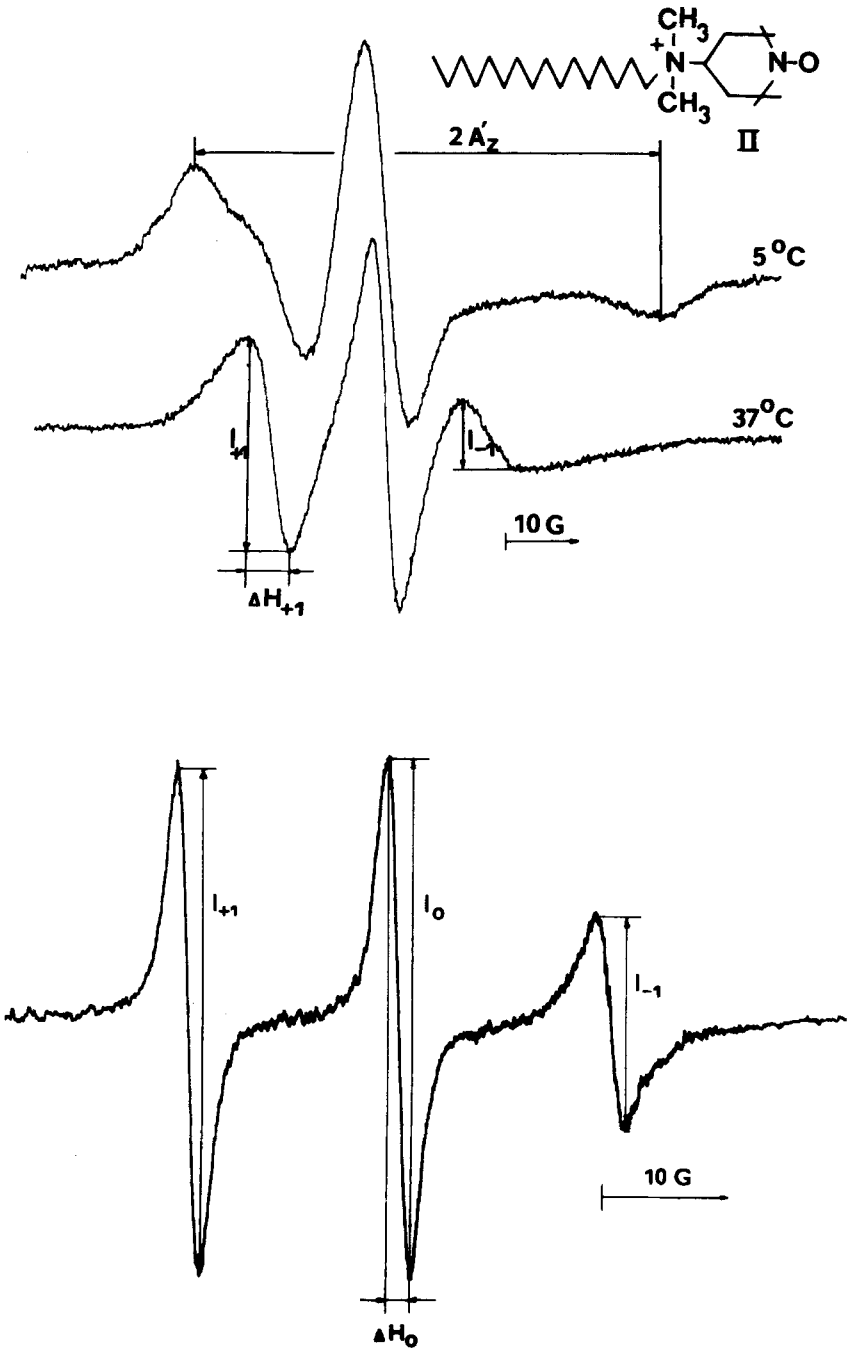

Fig. 2. Spin labeling of the surface coat of erythrocyte membranes. 2a - ESR spectra of the cationic spin probe CAT 16 incorporated into the erythrocyte membrane at different temperatures (150 mM NaCl, pH 7.4). 2b - ESR spectrum of covalently spin labeled sialic acid residues of glycophorin and glycolipids of the glycocalyx. The negative charged residues were labeled with TEMPAMINE (150 mM NaCl, pH 7.4, 37 °C). (Symbols - cf. Mat. and Meth.)

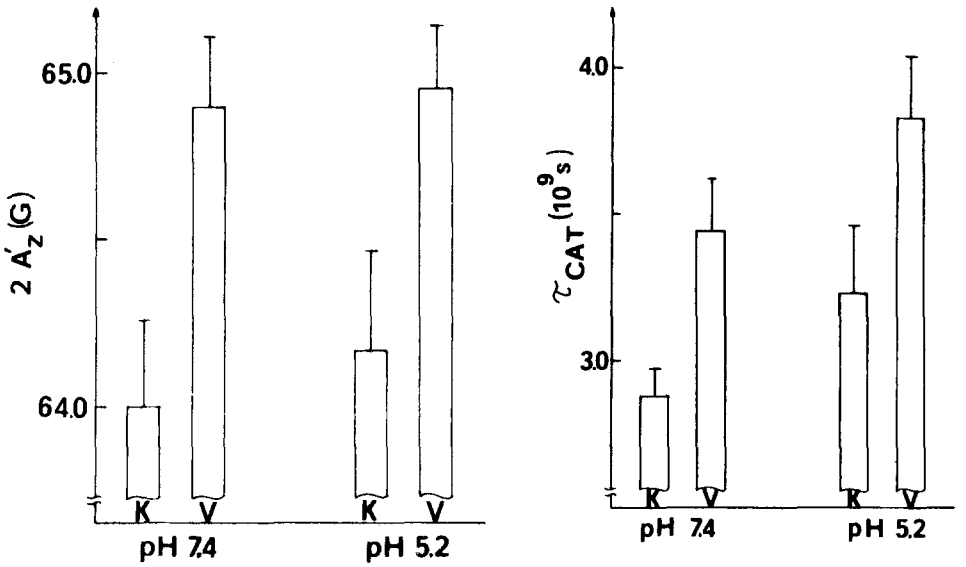

Fig. 3. External peak distance $2A_z'$ at 5 °C (Fig. 3a) and the correlation time τ_{CAT} at 37° (Fig. 3b) of the spin label CAT 16 incorporated into the erythrocyte membrane (150 mM NaCl, pH 7.4 and 5.2). K - control, V - after incubation for 10 min at 4°C in the presence of influenza virus (40 μg virus protein/ml). The standard error of estimate is given.

label is bound to residues of glycophorin, and 30% account for labeled glycolipids (Feix and Butterfield, 1980). The negative charge of sialic acid residues which serve as the receptor site for influenza virus (White *et al.*, 1983; Lyles and Landsberger, 1976) is preserved (Herrmann *et al.*, 1985). Both correlation times, τ_B and τ_C, respectively, were significantly enhanced after virus binding (also 40 μg virus protein/ml)—$\tau_B = (1.58 \pm 0.04)$ ns, $\tau_C = (1.86 \pm 0.04)$ ns—in comparison with the control sample—$\tau_B = (1.15 \pm 0.01)$ ns, $\tau_C = (1.41 \pm 0.01)$ ns (mean value ± SE of estimate, n = 3, pH 7.4, 20°C). The isotropic hyperfine splitting which is sensitive to the polarity of the surrounding medium was not influenced by virus attachment and was within the experimental error similar to that of the free label in aqua dest (16.8 G). Furthermore, no line broadening due to spin–spin interaction was observed.

Alterations of the Physical State of the Cytoskeleton

The spin label MAL-6 is covalently attached mainly to the spectrin network on the inner membrane surface of human erythrocytes (Barber *et al.*, 1985; Fung and Simpson, 1979). A typical spectrum of MAL-6 bound on membrane proteins is shown in Fig. 4a. The shape of the composite spectrum was already described (Fung and Simpson, 1979; Butterfield *et al.*, 1983; Fung, 1983; Butterfield and Markesberry, 1981). It consists of a strongly immobilized s_{+1} and a weakly immobilized (w_{+1}, w_{-1}) component. The amplitude ratio w_{+1}/s_{+1} was often used as a qualitative indicator of alterations in the physical state of membrane proteins. Since the ratio is very sensitive to differences between various blood samples and incubation conditions we have always compared the values before and after virus addition for each sample separately. In Fig. 4 the

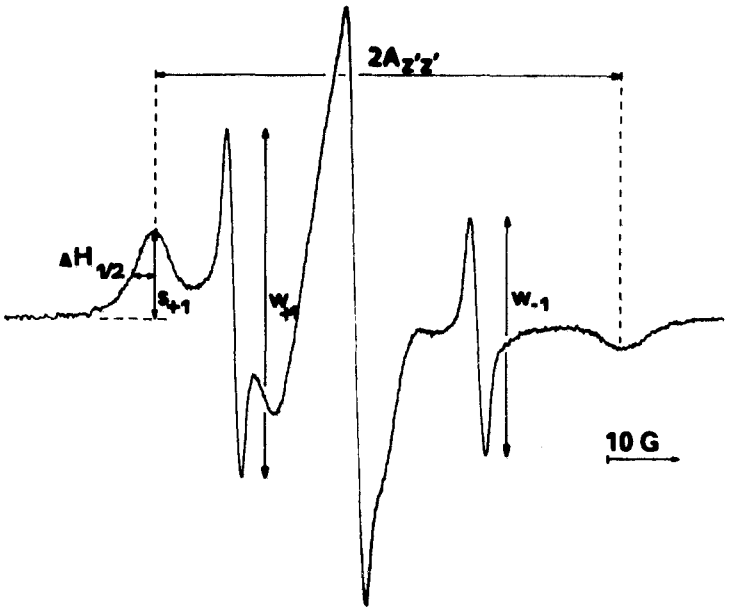

Fig. 4(a). ESR spectrum of MAL-6 covalently bound to membrane proteins of ghosts, mainly to the spectrin-actin meshwork at 20°C (150 mM NaCl, pH 7.4). w and s refer to amplitudes of the weakly and strongly immobilized component, respectively (cf. also Results).

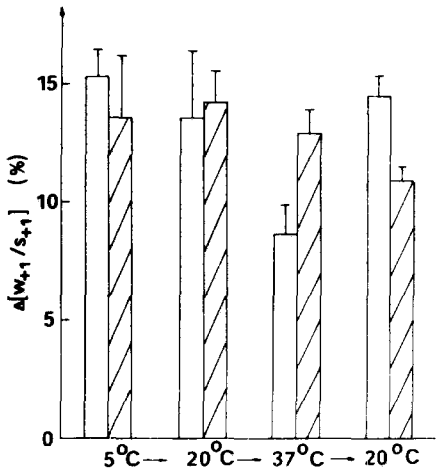

Fig. 4(b). Relative change of the amplitude ratio of the membrane spectrum of MAL-6 $\Delta w_{+1}/s_{+1}$ at different pH's after virus binding (40 μg virus protein/ml) at 4°C for 10 min. The spectra were recorded at different temperatures in the sequence indicated by the arrows. The standard error of estimate is given. ▨ - pH 5.2, □ - pH 7.4 (150 mM NaCl).

relative change of the ratio (co—control, vi—virus) $\Delta w_{+1}/s_{+1} = [(w_{+1}/s_{+1})_{co} - (w_{+1}/s_{+1})_{vi}]/(w_{+1}/s_{+1})_{co}$ at pH 5.2 is shown. In all cases we found a significant increase of the ratio, suggesting that virus binding caused alterations in the organization of the spectrin network. Since the virus is attached on the outer membrane surface, the observed effect was not due to a direct interaction between virus and the cytoskeleton. The absolute values of this ratio of the control were about 4 (pH 7.4) and

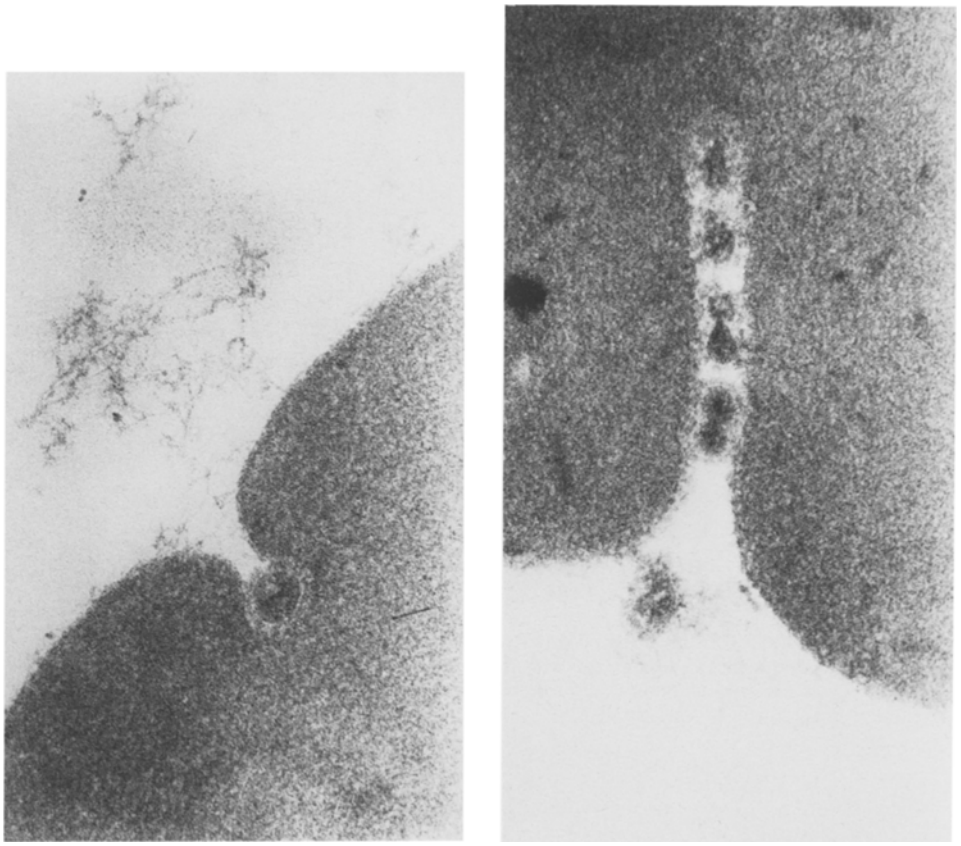

Fig. 5. Electron micrographs of influenza viruses attached to the human erythrocyte membrane. Samples were incubated at 4°C (10 min) and, subsequently, at 37°C (1 min). 150 mM NaCl, pH 5.2. Samples were prefixed with 2.5% glutaraldehyde, 1% formaldehyde in 0.1 M cacodylate buffer (pH 7.4). After replacing the fixative the membranes were embedded in 3% gelatine. The samples were then rinsed with the buffer and postfixed with 1% OsO_4 in the same buffer. After being rinsed in buffer the fixed membranes were embedded in 1% agar stained en bloc with 0.5% aqueous uranyl acetate, dehydrated in graded concentration of ethanol and embedded in Vestopal. Magnification: 100 000 ×. 5a - Invagination of influenze virus by the erythrocyte membrane. 5b - Tunnel-like invaginations are favored by local accumulation of viruses.

about 2 (pH 5.2) at 37°C in accordance with previous results (Fung, 1983; Butterfield and Markesberry, 1981). Virus binding did not influence the outer hyperfine splitting $2A_{Z'Z'}$ and the half line width $\Delta H_{1/2}$ of the strongly immobilized component.

Electron Microscopic Studies of Virus Binding

Electron microscopic studies were performed in order to detect morphological alterations of the plasma membrane immediately after virus attachment. A progressive encapsulation of the virus particle was established at pH 5.2 (37°C) (Fig. 5) causing locally a strong deformation of the plasma membrane. Tunnel-like invaginations were favored by a local accumulation of viruses. Similar pictures were obtained at pH 7.4 (37°C).

DISCUSSION

The positively charged spin probe CAT 16 is solely located in the outer leaflet of the human erythrocyte membrane (Laßmann and Herrmann, 1984). It is sensitive to steric as well as to electrostatic interactions with components of the surface coat, e.g. sialic acid residues of oligosaccharide chains (Laβmann and Herrmann, 1984; Herrmann *et al.*, 1985; Coughlin *et al.*, 1983; Hubbell, 1970). The mobility of the N–O group of CAT 16 was significantly reduced after virus adsorption on the cell membrane at both pH, 5.2 and 7.4, respectively. Since the membrane fluidity was not affected by virus binding, a direct interaction between viral glycoproteins and the N–O moiety of CAT 16 and/or an indirect influence on the label mobility caused by conformational alterations of the glycocalyx were assumed. Evidence for the latter was taken from the enhanced rotational correlation times of covalently spin labeled sialic acid residues of glycophorin and glycolipids reflecting also a more hindered mobility. These negatively charged groups are the attachment sites for the HA protein of the influenza virus (White *et al.*, 1983; Lyles and Landsberger, 1976). Because (a) the isotropic hyperfine splitting remained constant; (b) no spin–spin interaction was noted; and (c) no immobilized component in the ESR spectrum was detected in the presence of virus, we have no indication for any lateral reorganization of glycophorin and glycolipids, that means (I) no clustering as well as (II) no segregation out of cell membrane areas of virus contact. Protein-free lipid areas as a prerequisite of membrane–membrane fusion are still under discussion (Wilschut and Hoekstra, 1984). Lyles and Landsberger (1976) suggested a lateral reorganization of cellular virus receptors after virus binding. We rather suppose that the glycocalyx components at the contact site of the virus are fixed in their position with respect to the membrane surface. This might be accompanied by a conformational change of the molecular structure of the receptor. The presence and interaction of the sialic acid residues of the plasma membrane and the viral HA protein should be responsible for the chemical affinity of both membranes, allowing a stable aggregation and—as seen by electron microscopy—a progressive encapsulation of the virus particle by overcoming the work of deformation which competes with the chemical affinity (Evans and Parsegian, 1983; Evans and Buxbaum, 1981). It can be estimated that if the virus is embedded to a large extent the free energy contribution of the shear deformation increases dramatically in comparison with the bending resistance contribution (Evans and Parsegian, 1983; Evans and Buxbaum, 1981). It might be possible that the formation of an endocytotic vesicle is promoted by the unfavorable high curvature at the contact edges (Haywood and Boyer, 1981). Since we observed the invagination of the virus at both pHs, 5.2 and 7.4, respectively, we assume that encapsulation and virus–erythrocyte membrane fusion are triggered by different mechanisms although both functions reside on the HA-protein. Encapsulation of the virus was not accompanied with a virus-specific alteration of the fluidity of the erythrocyte membrane. Similar conclusions were drawn in case of interaction between Sendai viruses and erythrocyte membrane (Lyles and Landsberger, 1977, 1979).

Although the lipid phase was not affected, the physical state of the spectrin–actin meshwork was altered upon virus attachment at both pHs. Since the amplitude ratio used is only a qualitative measure we are not able to give detailed conclusions on the molecular alterations of the membrane proteins. The ratio does not permit us to

distinguish between changes of the conformational state and of the lateral organization of labeled proteins. However, the structural alterations of the cytoskeleton could be caused by (1) transmembrane signaling after virus binding *via* the structural linkage between glycophorin and spectrin initiating structural modifications (Wise, 1984) or (2) in response to the bending of the cell membrane during encapsulation. It might be speculated that in both cases the structural alterations of the cytoskeleton can favor or amplify the encapsulation of the virus. In that sense these results have interesting implications for an understanding of the virus endocytosis of influenza target cells by coated pits or vesicles, covered with the protein clathrin (Alberts *et al.*, 1983).

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