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# Isolation and Characterization of Erythrocyte Receptors for Measles Virus (40669)[1]

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Measles virus agglutinates monkey and baboon erythrocytes (1), but not erythrocytes of other species. Hemagglutination (HA) is effected through the interaction of the H spike glycoprotein in the viral envelope with receptors on the cell surface (2, 3). Little else is known about the receptor for this virus on either erythrocytes or susceptible cultured cells except that *N*-acetyl neuraminic acid (NANA) is not required for attachment. Indeed, neuraminidase treatment of erythrocytes enhances their susceptibility to measles viral HA, and exposure of Vero cells enhances slightly their susceptibility to infection (4). Previously presented data indicated that the receptor on erythrocytes is a glycoprotein which can be obtained in water-soluble form by treatment of membranes with lithium diiodosalicylate and phenol (LIS-ph) (5). We report here the characterization of simian erythrocyte glycoproteins and present data which point to their role as the receptors for measles virus.

*Materials and methods. Cells and virus.* Vero cells were grown to confluence in roller bottles in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Cells were washed twice with PBS and inoculated with the LEC strain of measles virus at a multiplicity of infection of 1 PFU/cell in Hanks' balanced salt solution. After adsorption the cells were overlaid with MEM containing 1% FCS. After 3 days, the cells were harvested and the culture fluid was clarified by centrifugation at 1200 *g* for 10 min. Clarified fluids were stored at −70° until used for purification of virus.

*Preparation of erythrocyte ghosts.* Rhesus monkey erythrocytes were obtained from Flow Laboratories and from the Delta Regional Primate Research Center, Covington, Louisiana. Packed erythrocytes in Alsever's citrate dextrose solution were washed and processed to hemoglobin-free membranes by the method of Dodge *et al.* (6). Hemoglobin-free ghosts were similarly prepared from sheep and human erythrocytes.

*Extraction of proteins from erythrocyte ghosts.* Proteins were extracted from erythrocyte ghosts according to the method described by Marchesi *et al.* (7). Briefly, lyophilized ghosts were mixed with a solution of 0.3 *M* LIS in 0.05 *M* Tris–HCl buffer, pH 7.5, at a concentration of 25 mg protein/ml. Water-soluble material was mixed with an equal volume of aqueous phenol and centrifuged at 4000 *g*. The aqueous phase was removed, dialyzed extensively against water, and lyophilized. Recovered proteins were washed with ethanol, relyophilized, and stored at 4°.

*Analysis of membrane proteins by polyacrylamide gel electrophoresis (PAGE).* Lyophilized erythrocyte membranes or LIS-ph-solubilized proteins (4 mg protein/ml) were mixed with water and treated with 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol (2-ME) in 0.05 *M* Tris–HCl, pH 6.8, and heated at 100° for 4 min. Particulate matter remaining in the solubilized mixture was removed by centrifugation at 2000 *g* for 10 min. Samples of approximately 100 μg protein (8) were analyzed in tube gels, using the discontinuous method (9), with either 7.5 or 9% separating gels and 3% stacking gels. Following electrophoresis, gels were stained by Coomassie blue (CB) or periodic acid Schiff (PAS) (10) methods or with resorcinol for sialic acid (11). Estimates of molecular weight were done in the same gel system, using, as standards, β-galactosidase, lactoperoxidase, bovine serum albumin (fraction V), catalase, glutamate dehydrogenase, fumarase, ovalbumin, pepsin, and trypsin.

*Isolation of proteins from gels.* Preparative gel electrophoresis was performed in slab gels

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using 3% stacking and 9% separating gels. A longitudinal slice with measured marks was cut from the gel and fixed and stained by the PAS method. Unstained portions of the gel corresponding to individual glycopolypeptides were excised, frozen, and minced with mortar and pestle. Gel fragments were stirred at room temperature with several changes of water and the slurry was then centrifuged to remove particulate matter. The supernatant was divided into two portions. One portion was lyophilized, resuspended in 5 ml of water, and dialyzed against 0.5 *M* Tris–HCl, pH 6.8. The other portion was dialyzed against five changes of 20% isopropanol, then dialyzed against water, lyophilized, resuspended in 5 ml of water, and finally dialyzed against Tris buffer.

*Assays for hemagglutination inhibition (HI).* Phosphate-buffered saline (PBS), pH 7.2, was used for dilutions and to suspend components in the assay. Serial twofold dilutions in PBS in volumes of 0.025 ml were made of LIS-ph-extracted proteins (1 mg/ml) from sheep, rhesus monkey, or human erythrocyte ghosts. Eight hemagglutinating units (HAU) of purified measles virus in 0.025 ml of PBS were added to each well, mixed, and incubated for 30 min at 37°. Twenty-five microliters of washed monkey erythrocytes was added to each well of the dilution series. The plates were incubated at 37° for 1 hr and HI titers were determined.

HI titers of individual glycosylated polypeptides of erythrocyte ghosts were determined, after elution from preparative gels and extensive dialysis against 20% isopropanol and PBS. The amount of SDS remaining in the dialyzed preparations was estimated by titering hemolytic activity compared with that of solutions of known concentrations, in order to establish the necessary controls for HI assays. Eluted glycoproteins in 0.025 ml were serially diluted and 8 HAU of virus in 0.025 ml (were serially diluted and 8 HAU of virus in 0.025 ml) was added to each dilution. Bovine serum albumin was added to the mixtures to a final concentration of 1%, followed by 0.025 ml 1% rhesus monkey erythrocytes fixed in 0.05% glutaraldehyde (12).

*Adsorption of membrane glycoproteins to purified measles virus.* Measles virus was purified by previously published methods (13). Twenty-five microliters of LIS-ph-extracted proteins (500 μg/0.5 ml) were mixed with ~20,000 HAU/ml of purified measles virus and incubated at 37° for 1 hr. The mixture was layered onto 1.5 ml of 20% (w/w) sucrose in PBS and centrifuged at 189,000 *g* for 45 min in the SW 50.1 rotor. The supernatant was removed and the virus pellet was suspended in PBS and recentrifuged in the type 30 rotor at 50,000 *g* for 75 min. LIS-ph-extracted proteins without virus were layered over the sucrose barrier and processed in an identical manner. The virus plus adsorbed proteins were then solubilized and analyzed by PAGE.

*Results. PAGE analysis of erythrocyte ghost proteins.* Rhesus monkey erythrocyte ghosts were solubilized with SDS and 2-ME and the proteins were analyzed by PAGE. Profiles of erythrocyte membrane glycopolypeptides and polypeptides stained with PAS and CB are shown in Fig. 1. Five components stained with PAS (Fig. 1A) which had approximate molecular weights, respectively, of 120K (PAS 1), 90K (PAS 2), 64K (PAS 3), 48K (PAS 4), and 33K (PAS 5). PAS 3 and 5 consistently gave a lighter stain than PAS 1, 2, or 4. The positions of bands stained with CB (Fig. 1B) did not correspond with those stained with PAS. CB bands 1 and 2 were high-molecular-weight polypeptides of ap-

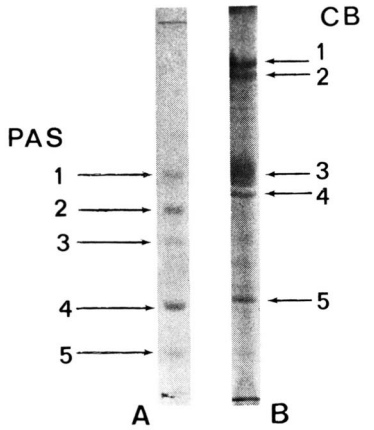


FIG. 1. PAGE analysis of membrane proteins from rhesus monkey erythrocytes. Membranes were treated with SDS and 2-ME and heated at 100° for 4 min. 100 μg of each protein was electrophoresed in SDS–disc gels. Gels A and B were stained by PAS and CB methods, respectively.

proximately 280K and 240K $M_r$, respectively. CB band 3 was diffuse, possibly consisting of several components as suggested by the gradation of staining. Several minor bands stained with CB in the regions between CB 2 and 3, CB 4 and 5, and ahead of CB 5.

*PAGE analysis of LIS-ph-extracted glycoproteins of erythrocyte ghosts.* Proteins solubilized by LIS-ph treatment of rhesus monkey, human, and sheep erythrocyte ghosts were analyzed by PAGE and stained by the PAS method. Additional bands were evident in the LIS-ph-extracted preparations of rhesus monkey erythrocytes (Fig. 2B) compared to those seen in the gels of membranes directly solubilized with SDS and 2-ME (Fig. 2A). Moreover, in profiles of LIS-ph glycoproteins there was a close correspondence between PAS- and CB-stained bands (not shown) in contrast to the dissimilarity between CB- and PAS-stained bands of whole membranes (Fig. 1). All LIS-ph-extracted polypeptides were also stained by the resorcinol method (11) indicating the presence of NANA (not shown). Polypeptides of human and sheep erythrocytes were extracted and analyzed similarly. On comparison, the multitude of high-molecular-weight bands found in gels of rhesus monkey preparations were not found in extracts of human and sheep erythrocytes. One minor and two major bands were seen above the four glycopolypeptides (PAS 1–4) found in human erythrocyte ghosts (Fig. 2D). In LIS-ph preparations of sheep erythrocyte membranes four diffuse bands migrated behind PAS 1, 2, and 3 (Fig. 2G), the latter glycopolypeptides being found solely in ghosts (Fig. 2F). PAS 1 was enriched compared to its counterpart in ghost profiles.

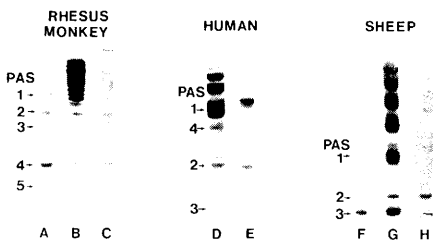


FIG. 2. PAGE analysis of glycoproteins (PAS stained) of erythrocyte membranes and their binding to measles virus. Rhesus monkey and sheep erythrocyte membranes free of hemoglobin were treated with SDS and 2-ME and heated at 100° and soluble proteins were analyzed (A and F, respectively). Rhesus monkey, human, and sheep erythrocyte membranes were also treated with LIS-ph and the water-soluble proteins were denatured with SDS and 2-ME by heating (B, D, G, respectively). Proteins extracted with LIS-ph were mixed with purified virus and incubated, and virus was then centrifuged through a sucrose barrier. Viral proteins and erythrocyte membrane proteins adhering to virus were solubilized (C, E, H).

*Interconversion of PAS-positive polypeptides.* Rhesus monkey erythrocyte membranes were solubilized with SDS and 2-ME and electrophoresed in preparative slab gels. Bands PAS 1 to 5 were located, eluted from gel slices, and dialyzed against either isopropanol or buffer as described under Materials and Methods. PAGE analysis of the eluted bands which had not been dialyzed against isopropanol yielded bands in the same position as before isolation. However, as shown in Fig. 3 bands dialyzed against isopropanol were resolved into bands of higher and lower molecular weight in addition to the original band. Eluted PAS 1 yielded not only PAS 1, 2, and 4, but also three bands migrating more slowly than PAS 1 and designated a, b, and c. PAS 2 yielded PAS 1, 2, and 4, small amounts of PAS 3 and 5, and bands b and c. PAS 3 yielded PAS 1, 2, 3, 4, and 5 and band c. Band PAS 4 gave rise to PAS 1, 2, and 4. PAS 5 yielded PAS 1 and 3, but no detectable PAS 5.

*Hemagglutination inhibition of LIS-ph-extracted proteins and isolated glycopolypeptides from erythrocyte membranes.* LIS-ph-extracted proteins from human, rhesus monkey, and sheep erythrocyte membranes were tested for inhibition of hemagglutination by measles virus. As shown in Table I, only LIS-ph-extracted proteins of rhesus monkey erythrocytes significantly inhibited hemagglutination, this fraction being at least 10 × more potent than similar fractions of human or sheep erythrocytes. Glycopolypeptides from rhesus monkey ghosts (PAS 1–5) each inhibited the agglutination of glutaraldehyde-fixed homologous erythrocytes.

*Adsorption of LIS-ph-extracted proteins to purified measles virus.* Lis-ph-extracted proteins from sheep, rhesus monkey, and human erythrocytes were mixed with purified measles virus and incubated at 37°. Following

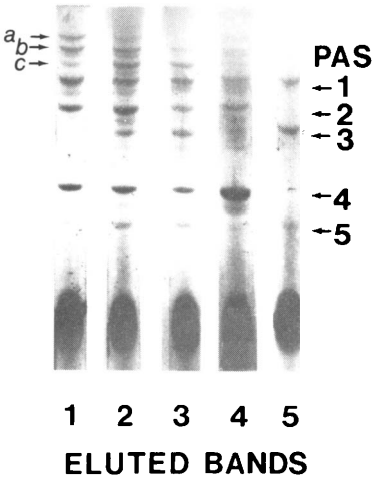


FIG. 3. Relationship between individual glycopolypeptides from membranes and those from LIS-ph-extracted material. Membrane ghosts were treated with SDS and 2-ME and electrophoresed in preparative SDS-disc slab gels. Bands PAS 1 through 5 were located and individually sliced from the gel and eluted into water. Eluates were dialyzed against 20% isopropanol and then 0.05 *M* Tris buffer. Each band preparation was reelectrophoresed (50–75 μg protein/gel) in SDS tube gels and stained with PAS. Gels 1–5 correspond to the individual band eluted from preparative gels. PAS 1 through 5 indicate the position of each PAS band from membranes.

TABLE I. HI ACTIVITY OF ERYTHROCYTE GLYCOPROTEINS

| Fraction | μg inhibiting 8 HA units measles virus |
|---|---|
| LIS-ph glycoproteins | |
| Human | >108 |
| Rh Monkey | 12 |
| Sheep | >110 |
| PAGE eluates | |
| Rh monkey | |
| PAS 1 | 11 |
| PAS 2 | 10 |
| PAS 3 | 19 |
| PAS 4 | 8 |
| PAS 5 | 23 |
| Human glycophorin A | >50 |

incubation, the virus was centrifuged through a sucrose barrier and washed once. The virus pellets were then analyzed by PAGE. LIS-ph-extracted material alone served as control and no pellet was recovered. As shown in Fig. 2C, only two major glycopolypeptides from the total LIS-ph-extracted proteins of rhesus monkey erythrocytes (Fig. 2B) adsorbed to measles virus. The two bands corresponded in electrophoretic migration to PAS 2 and 4 of the polypeptide profile of membrane (Fig. 2A). In addition, several lightly staining bands were evident migrating above and below PAS 2.

LIS-ph-extracted proteins from human and sheep erythrocyte membranes were adsorbed with purified measles virus in similar manner. Adsorbed virus was analyzed by PAGE along with the LIS-ph-extracted proteins. Of the total human erythrocyte LIS-ph-extracted proteins (Fig. 2D) the upper, most slowly migrating portion of PAS 1 adsorbed to the virus (Fig. 2E). In addition, PAS 2 and 3 and a trace of PAS 4 adsorbed to the virus. Aggregated glycopolypeptides which migrated behind PAS 1 did not adsorb to virus under these conditions. Of the six PAS bands of sheep erythrocyte glycoproteins, only PAS 2 adsorbed to measles virus to any appreciable degree (Fig. 2H).

*Discussion.* The glycoproteins extracted from membranes of rhesus monkey erythrocytes inhibited measles virus-mediated agglutination of homologous erythrocytes. Bands corresponding to PAS 2 and 4 of the PAGE profile of erythrocyte ghosts were preferentially adsorbed by purified virus. Aggregated glycoproteins were not found with the viral pellet and no glycoproteins by themselves in the absence of virus passed through the sucrose barrier. These facts argue for some degree of specificity in this interaction. Data to suggest that certain of the glycoproteins in LIS-ph extracts of simian erythrocytes act as receptors in intact membranes were obtained by analysis of individual glycopolypeptides eluted from preparative PAGE. Each band (PAS 1–5) by itself inhibited agglutination. PAGE analyses of eluted glycoproteins after dialysis against isopropanol yielded in each case not only the original bands, but also aggregated forms of higher and lower molecular weights not previously present as such. The final patterns of glycopolypeptides so derived from each of the original eluted bands were all very similar to one another, except for that of PAS 5, which in contrast to the others showed the least specific activity. After dialysis, PAS 4 gave rise to PAS 1 and 2, and PAS 5 yielded PAS 1 and 3. PAS 4 and 5 may therefore be monomers which

differ in structure and which form correspondingly diverse aggregates. Accordingly, PAS 2 and 4 were preferentially adsorbed to purified virus and had higher specific antiviral activity than PAS 5. This suggests that PAS 4 and/or its derivatives serve as the chief receptors for measles virus, PAS 5 having only limited activity.

Although neither human nor sheep erythrocytes are agglutinated by measles virus, soluble glycoproteins of either species were specifically adsorbed to virus. Of the six sheep cell polypeptides, only one was reactive with virus. From the analogous human material, several components appeared to be specifically adsorbed. Most prominent was the slowest portion of the diffuse PAS 1 (glycophorin) band. The two polypeptide bands with molecular weights higher than glycophorin (Fig. 2D) did not adsorb. PAS 2 and 3, however, did adsorb to virus. This may be explained by the fact that PAS 1 represents aggregates of the lower-molecular-weight glycopeptides, chiefly PAS 2 and 3 (14) which may, therefore, have the same specificity for virus as the reactive portion of PAS 1, and which is not a property of the components larger than PAS 1.

As pointed our earlier, receptors on sheep and human erythrocytes possibly because of steric considerations, are not accessible to viral hemagglutinin, yet certain of the glycoproteins solubilized from membranes are selectively reactive with virus. In order to explain this apparent discrepancy, one could surmise that adsorption of soluble receptor glycoprotein by virus is a multiphase process, which with the sheep and human material does not interfere with attachment of viral HA to intact receptors on simian erythrocytes.

*Summary.* Glycoproteins extracted in water-soluble form from rhesus monkey erythrocytes were shown to inhibit hemagglutination by measles virus. PAGE analysis of glycoproteins from erythrocyte membranes and in LIS-ph extracts showed that lower-molecular-weight glycoproteins readily formed aggregates which resisted dissociation with SDS and 2-ME at 100°. Preferential binding of isolated glycoproteins of erythrocyte membranes to purified virus was demonstrated even though all of these inhibited hemagglutination to some extent. Data presented here suggest that specific forms or configurations of membrane glycoproteins constitute receptors for measles virus.

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