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Labelling of membrane glycoprotein in erythrocytes infected with *Plasmodium knowlesi* *

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Normal rhesus monkey erythrocytes and erythrocytes infected by P. knowlesi *were labelled with galactose oxidase (EC 1.1.3.9) and tritiated sodium borohydride. The glycoproteins of normal erythrocytes were not labelled unless the cells were pretreated with neuraminidase, when peaks of activity with apparent molecular weights of 170 000, 126 000, 90 000, 50 000, and 35 000 were observed. Schizont-infected erythrocytes showed an absence of glycoprotein labelling even after neuraminidase treatment. The results indicate that there is an alteration in the glycoproteins of schizont-infected erythrocytes, which may contribute to the increased permeability and the immunological alterations on the surface of these cells.*

As the malarial parasite grows from the ring to the schizont stage within the host erythrocyte, the erythrocyte changes its shape from a biconcave disc to a sphere with surface crenations (*1*). In the human erythrocyte the biconcave cell shape, the resistance to deformation, and other physical properties of the intact cell are controlled by a network of spectrin, probably in conjunction with actin-like component V (*2*). The intracellular growth of *Plasmodium berghei* (*3*) and *P. chabaudi* (*24*) results in the degradation of the spectrin of the host erythrocyte, which would account for the morphological changes in infected cells. These alterations in the structural arrangement of the erythrocyte membrane could indirectly account for changes in electrolyte transport (*4, 5*), amino acid transport (*6, 7*), and permeability to L-glucose (*8*) in malaria-infected erythrocytes.

Alterations in the membrane proteins of erythrocytes infected with *P. knowlesi* have been detected by sodium dodecyl sulfate (SDS) gel electrophoresis and iodine-125 lactoperoxidase labelling (*25*) and by concanavalin A binding (Shakespeare et al., in preparation). In this paper we report on the application of the method of Gahmberg & Hakomori (*9*), using galactose oxidase and tritiated sodium borohydride to analyse the surface carbohydrates of normal rhesus monkey erythrocytes and erythrocytes infected with schizonts of *P. knowlesi*.

MATERIALS AND METHODS

Preparation of cells

P. knowlesi (Nuri strain) was maintained in male and female rhesus monkeys (*Macaca mulatta*) by weekly syringe passage of infected blood, or as frozen stabilates of infected blood in 10% glycerol stored at −70°C. Infected monkeys were bled when parasitaemia was 30% or more and when the parasites were at the schizont stage. Both normal and schizont-infected cells were prepared from freshly drawn heparinized blood (10 parts blood to 1 part heparin/Ringer solution, giving a final concentration of 17 units/ml). Centrifugation at 1500 *g* for 10 min was followed by washing twice under the same conditions with Krebs glucose saline. The erythrocytes were separated from white cells and platelets by means of a sucrose gradient (*10*) or by means of a combination of a column of CF 11 cellulose (*11*) and a sucrose gradient.

Chemicals

Tritiated sodium borohydride (specific activity 10.84 GBq/mmol) was obtained from the Radiochemical Centre, Amersham, England. Neuraminidase (EC 3.2.1.18) and galactose oxidase (EC

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1.1.3.9) were purchased from Sigma Chemicals. All other chemicals were of analytical reagent grade.

Treatment of cells with neuraminidase

The washed cells were suspended in twice their volume of 0.1 mol/litre sodium phosphate buffer, pH 6.0, and 0.05 ml of the neuraminidase solution (5 units/ml in 0.1 mol/litre phosphate buffer, pH 6.0) per ml of packed cells was added. The cells were incubated at 37°C for 30 min with continuous shaking, then transferred to an ice–water bath and washed three times by centrifugation at 1500 *g* for 10 min with a large volume of ice-cold phosphate-buffered saline (PBS), pH 7.0.

Labelling of cells

Normal and infected cells were labelled, using galactose oxidase and tritiated sodium borohydride, by the method of Gahmberg & Hakomori (*9*). Cells treated with neuraminidase were similarly incubated. After collection, the cells were washed three times in PBS, pH 7.0, by centrifugation at 1500 *g* for 10 min and 40 μg of galactose oxidase dissolved in PBS, pH 7.4, were added to each 1 ml of packed cells. The cells were incubated for 3 h at 37°C in a water bath with gentle shaking and were then washed twice with PBS, pH 7.4, by centrifugation at 1500 *g* for 10 min. Freshly prepared tritiated sodium borohydride solution (0.05 ml) was added to the washed erythrocytes at room temperature. The sodium borohydride solution was prepared by dissolving 37 MBq of the substance in 0.2 ml of PBS, pH 7.4. After incubation at room temperature for 30 min with occasional shaking, 1 mg of unlabelled sodium borohydride was added and the cells were diluted with 5 ml PBS, pH 7.4, and shaken well.

Preparation of membranes

After labelling, the cells were lysed by the addition of four volumes of cold distilled water, followed by mixing with a vortex mixer. The lysate was then centrifuged at 30 000 *g* for 30 min and the pellet washed once by resuspension and recentrifugation in 5 mmol/litre Tris-chloride buffer, pH 7.4. These conditions were shown to sediment all the erythrocyte-bound acetylcholinesterase (EC 3.1.1.7) (Shakespeare et al., in preparation).

Gel electrophoresis

Separation of labelled protein species was performed by electrophoresis on cylindrical 7.5% acrylamide gels in the presence of sodium dodecyl sulfate (SDS) by the method of Laemmli (*12*). Membrane proteins were made soluble in the dissociating medium by heating them in boiling water for 2 min. Each gel was loaded with a protein concentration (about 400 μg), which was derived from a similar number of cells, and separation was performed at 3 mA per gel (7 mm internal diameter) until the dye front had reached the end of the tube. The gels were frozen in dry ice and sliced transversely into slices of approximately 1 mm, using a stack of razor blades with spacers. Each gel slice was dissolved in 0.3 ml NCS overnight at room temperature and, after the addition of 6 ml toluene-based scintillation fluid (3.5 g PPO and 50 mg POPOP per litre), radioactivity was counted in a Packard Tri-Carb 2450 liquid scintillation counter. The efficiency of tritium counting was approximately 30%.

Molecular weights were calculated from a standard curve using ceruloplasm (151 000), bovine serum albumin (68 000), pyruvate kinase (EC 2.7.1.40) (57 000), ovalbumin (46 000), alcohol dehydrogenase (EC 1.1.1.1) (42 000), lactate dehydrogenase (EC 1.1.1.27) (35 000), chymotrypsin (EC 3.4.21.1) (25 000), myoglobin (17 800), and cytochrome c (12 400) as standards.

RESULTS

In the absence of galactose oxidase, there was no significant nonspecific binding of tritiated sodium borohydride to membrane proteins of both untreated (Fig. 1, graph A), and neuraminidase-treated (Fig. 1, graph B) normal monkey erythrocytes and erythrocytes infected by *P. knowlesi*.

Galactose oxidase failed to stimulate the labelling of proteins of both untreated normal and infected monkey erythrocytes, although some labelling of lipids was observed (Fig. 1, graph C) as a peak just behind the tracking dye. However, control samples of normal human erythrocytes incubated with galactose oxidase did show marked stimulation with several peaks, with apparent molecular weights ranging from 168 000 to 37 000, a pattern of labelling similar to that described by Gahmberg & Hakomori (*9*). These results probably indicate that the monkey erythrocyte has fewer glycoproteins with exposed galactose and *N*-acetylgalactosamine residues on its external surface than has the human erythrocyte.

Pretreatment of infected monkey erythrocytes with neuraminidase, followed by incubation with

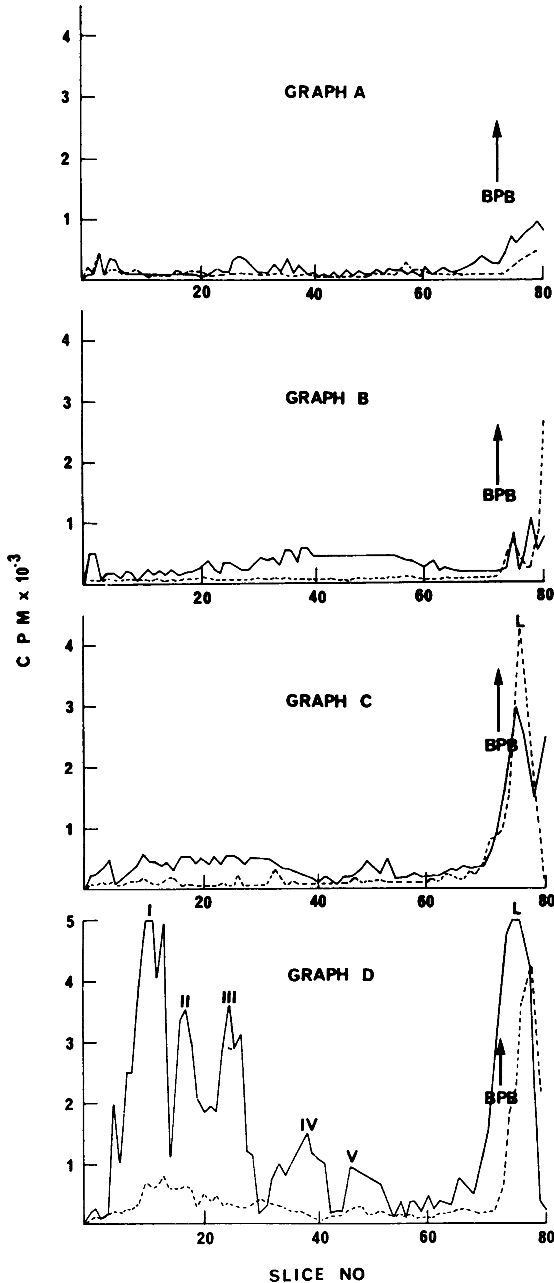

Fig. 1. Galactose oxidase–tritiated borohydride labelling of normal erythrocytes and erythrocytes infected with schizonts of *P. knowlesi*. The solid line represents normal erythrocytes, the broken line infected erythrocytes. Graph A = control; graph B = control, neuraminidase treated; graph C = galactose oxidase treated; graph D = galactose oxidase and neuraminidase treated. BPB = bromophenol blue.

galactose oxidase and tritiated borohydride, also failed to stimulate labelling of other proteins or lipids. However, normal monkey erythrocytes treated with neuraminidase did show significant binding of radioactivity to proteins with several peaks of activity in species with apparent molecular weights of 170 000 (peak I), 126 000 (peak II), 90 000 (peak III), 50 000 (peak IV), and 35 000 (peak V) (Fig. 1, graph D). These results imply that there are at least qualitative if not quantiative differences in the glycoproteins of normal erythrocytes and erythrocytes infected with schizont stages of *P. knowlesi*.

DISCUSSION

It has been shown that, in intact cells, the labelling of galactosyl and galactosamine residues of human erythrocytes, by oxidation with galactose oxidase followed by reduction with tritiated borohydride, can be assigned to the residues on the external surface of the membrane (*9*, *13*). Rhesus monkey erythrocytes differ markedly from those of humans as the galactose oxidase–borohydride labelling of monkey cells is negligible unless the cells have been pretreated with neuraminidase. A similar situation has been observed in equine erythrocytes (*14*) and this apparent inaccessibility of the monkey and equine glycoproteins may be due partly to their structure and partly to their localization within the erythrocyte membranes. The difference in labelling characteristics may not be too surprising since it is well known that although polypeptide profiles of erythrocytes from different mammalian species closely resemble those from man, the glycoproteins are more diverse (*15*, *16*, *17*). In addition, the staining intensity of proteins from normal monkey erythrocytes, separated on SDS-polyacrylamide and stained by the periodic acid-Schiffs method, has been shown to be much fainter than those of similarly prepared membranes from human erythrocytes (*18*).

After neuraminidase treatment, normal monkey erythrocytes labelled by the galactose oxidase method showed peaks of activity with apparent molecular weights of 170 000, 126 000, 90 000, 50 000, and 35 000. These peaks were absent in erythrocytes infected with schizont stages of *P. knowlesi*. We have not identified these labelled peaks further but since sialoglycoproteins are known to contain galactose in the terminal as well as the penultimate positions (*19*, *20*) they are susceptible

to attack by galactose oxidase. The results obtained by treatment of the normal cells by neuraminidase are consistent with this hypothesis.

Although galactose oxidase treatment may result in major labelling of components that are only minor as detected by protein and glycoprotein stains (*13*), the results in this paper may possibly be correlated with recent observations on changes in the membranes of erythrocytes infected with *P. knowlesi*. The loss of component labelling with an apparent molecular weight of 126 000 may correspond with changes observed in the band III protein (120 000) found in normal monkey erythrocyte membranes. This band III contains the receptor for concanavalin A (*21*) and schizont-infected erythrocytes possess fewer binding sites for concanavalin A than normal monkey erythrocytes (Shakespeare et al., in preparation). Band III also contains a protein (I-1a), which is labelled by ^{125}I-lactoperoxidase but which is lost in erythrocytes containing schizonts of *P. knowlesi* (*25*). Band III also contains galactose (*22*), which would be susceptible to labelling by the galactose oxidase–borohydride method. The loss of the 90 000 and 50 000 peaks may correspond to changes observed by Wallach & Conley (*25*) in band IV (109 000) and Band V (52 000) of membranes from *P. knowlesi*-infected cells. These authors showed that band IV includes a glycoprotein, PAS 1a, and an iodine-stainable component, I-1b, the former component being modified in infected cells. Band V was shown also to contain two components, one a glycoprotein (PAS 3) and the other an iodine-stainable component (I-3), the latter of which was either reduced or deleted in infected cells. The radioactive peak of apparent molecular weight 35 000 corresponds to band VI on protein stained gels of membranes from normal monkey erythrocytes (*25*). Similar bands of radioactivity with apparent molecular weights of 85 000, 52 000, and 35 000 have been observed in normal human erythrocytes following neuraminidase treatment and labelling with galactose oxidase–borohydride (*9*).

The labelling of the 170 000 peak in normal erythrocytes was highly variable and it is possible that the neuraminidase treatment of our cells had resulted in the labelling of a component on the inside of the erythrocyte membrane. A component with similar molecular weight 150 000, which was attributed to the inner surface, has been described in the labelling of erythrocyte ghosts by galactose oxidase–borohydride (*9*).

The alterations in glycoproteins described here may be involved in the changes in the permeability of the infected cell, since the major glycoproteins are associated with the " permeaphore " structure, which is concerned with transport and water movement (*2*). They may also account for the alteration in the surface of schizont-infected erythrocytes detected by immunological techniques (*23*).

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RÉSUMÉ

MARQUAGE DES GLYCOPROTÉINES DE LA MEMBRANE SUR DES ÉRYTHROCYTES INFECTÉS PAR *PLASMODIUM KNOWLESI*

Des érythrocytes normaux de singes rhésus et des erythrocytes infectés par *Plasmodium knowlesi* ont été marqués à l'aide de galactose oxydase et de borhydrure de sodium tritié. Les glycoprotéines et les érythrocytes normaux n'ont été marqués qu'après traitement des cellules à la neuraminidase, alors que l'on observait des pics d'activité, avec des poids moléculaires apparents de 170 000, 126 000, 90 000, 80 000 et 35 000. On a constaté dans les érythrocytes infectés par les schizontes une absence de marquage des glycoprotéines, même après traitement à la neuraminidase. Ces résultats démontrent une altération des glycoprotéines des érythrocytes infectés par les schizontes — phénomène qui peut contribuer à accroître la perméabilité et aggraver les altérations immunologiques de la surface de ces cellules.

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