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Labelling of membrane glycoproteins of cultivated *Plasmodium falciparum**

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Membrane glycoprotein synthesis by Plasmodium falciparum *was determined by metabolic labelling in the presence of 74 kBq/ml (2.5 μCi/ml) glucosamine-^{3}H. Five major glycoprotein bands and four minor ones were demonstrated. A control experiment using normal, outdated, human erythrocytes indicates that there was no incorporation of the labelled glucosamine into the erythrocyte membrane. It was also demonstrated that the rate of membrane glycoprotein synthesis by mature parasites of the trophozoite and schizont stages was twice that of the ring-stage parasites. Cytochemical surface-labelling experiments had led to the conclusion that the membrane of malaria parasites contains little or no glycoprotein. Our studies indicate, however, that there is significant synthesis of membrane glycoprotein by the parasite and that this can be metabolically labelled and measured by using radioactive glucosamine as precursor of the glycoprotein.*

Malaria caused by *Plasmodium falciparum* is a major cause of death, particularly of children, in many countries of Africa, Asia, and South America. Death is usually associated with a massive destruction of erythrocytes in the infected person. The first step in the invasive process involves an attachment of the parasite to the exterior of the erythrocyte, the parasite probably attaching itself through receptors located on its cell coat. Although the chemical make up of the cell coat has not been fully determined, it has been shown to contain a glycoprotein (*1*). Since the cell adhesion factor located on the surface of fibroblasts which mediates their attachment and spread (*2*) is a glycoprotein, it is possible that the attachment of malarial parasites to erythrocytes is also mediated through a glycoprotein. This possibility emphasizes the need for a better understanding of the biology of membrane and cell-coat glycoproteins in order to evaluate their role in the mechanisms of parasite attachment and invasion.

Most studies of glycoproteins in malarial parasites have used cytochemical staining and other techniques which, in general, detect glycosides located only on the outer surface of cells. These experiments have led to the conclusion that the parasite membrane contains little, or no, glycoprotein (*3*, *4*). In contrast to those studies, however, we now report a significant incorporation of glycoprotein precursors, glucosamine-^{3}H, fucose-^{3}H, and mannose-^{14}C into membrane glycoproteins of *P. falciparum*, cultivated *in vitro*.

MATERIALS AND METHODS

Culture methods

Erythrocyte-stage parasites (kindly supplied by Dr B. J. Jensen and Professor W. Trager, Rockefeller University, New York, USA) were maintained in continuous culture (as parasitized erythrocytes) in our laboratory using the candle jar culture methods of Jensen & Trager (*5*).

Labelling and membrane preparations

Parasitized erythrocytes were incubated at 37 °C in the presence of 74 kBq/ml (2.0 μCi/ml) of glucosamine-^{3}H, or fucose-^{3}H, or mannose-^{14}C[a] for different periods then centrifuged at 495 *g* for 10 min. The pellet was resuspended and washed three times in 10 ml of iso-osmotic phosphate buffer, pH 7.4, to remove any unincorporated radioactivity. "Free parasites" were obtained by lysing the washed parasitized erythrocytes with Trager's high potassium buffer containing 0.03% saponin, following which the lysate was passed through a 0.45-mm (26-gauge) needle several times to remove any erythrocyte fragments that might be clinging to the freed parasites. This method has been demonstrated to yield parasites essentially free of erythrocyte-membrane material (B. J. Jensen, personal communication 1978). After passing the lysate through the 0.45-mm needle, it was

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centrifuged at 500 *g* for 10 min. The pellet containing the free parasite was resuspended in an equal volume of buffer, and gently layered on top of an equal volume of 5% bovine serum albumin (BSA) in Trager's buffer. This was then centrifuged at 4430 *g* for 20 min. The resulting supernatant, which contained the erythrocytic-membrane fragments, was discarded. The pellet containing the purified free parasites was resuspended and the parasites were lysed using 10 volumes of hypotonic phosphate buffer, pH 7.4. The lysate was centrifuged at 100 000 *g* for 30 min and the resulting pellet containing parasite-membrane fragments was washed once more. The washed-membrane fragments were resuspended in 1 ml of iso-osmotic buffer and stored at −22 °C until used. In control experiments, nonparasitized erythrocytes were incubated with glucosamine-^{3}H, fucose-^{3}H, or mannose-^{14}C under the same experimental conditions as the parasitized erythrocytes. Membranes were prepared according to the methods of Dodge et al. (*6*) and stored at −22 °C until used.

Electrophoretic methods

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the methods of Laemmli (*13*). About 50 μg of the parasite fragments were applied to the rod gels and electrophoresis was carried out with a current of 8 mA/gel until the tracking dye reached the end of the gel. BSA, fructose-biphosphate aldolase (EC 4.1.2.13.), lysozyme (EC 3.2.1.17.), and carbonate dehydratase (EC 4.2.1.1.) were used as standards. At the end of the run, the gels were crushed (1-mm lengths) in a Gilson gel crusher and eluted in 15 ml of 3% Protosol[a] in Econofluor and radioactivity was determined in a Packard Liquid Scintillation Counter.

RESULTS AND DISCUSSION

Glucosamine is a precursor for *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, and sialic acid residues of glycoprotein (*7*). The addition of 74 kBq/ml of this precursor to the incubation mixture resulted in a significant incorporation of radioactivity into membrane glycoproteins from free parasites, with only minor amounts of glucosamine-^{3}H being incorporated into the membranes of erythrocytes (Table 1). Total radioactive incorporation increased linearly with increasing time of incubation. The rate of precursor incorporation into glycoprotein appeared to depend on the developmental stage reached by the parasites (Fig. 1). The "A" population, which consisted of 81% mature trophozoites and schizont stages and 19% rings showed twice the rate of incorporation shown by the "B" population, which consisted of 90% rings and 10% trophozoites.

[a] From New England Nuclear, Boston, MA, USA.

Table 1. Incorporation of labelled carbohydrates into membrane glycoproteins in *P. falciparum* and unparasitized erythrocytes[a]

Precursor	Membrane type	Disintegrations per min (dpm) per mg of protein[b]	Amount of precursor incorporated (nmol/mg of protein)[c]
glucosamine-^{3}H	membrane from free parasites	118 000.0	1.3
	unparasitized erythrocyte membrane	400.0	ND
fucose-^{3}H	membrane from free parasites	16 000.0	0.1
	unparasitized erythrocyte membrane	500.0	ND
mannose-^{14}C	membrane from free parasites	19 000.0	0.03
	unparasitized erythrocyte membrane	300.0	ND

[a] Incorporation was determined from 0.2 ml of membrane suspension. 0.5 ml of Protosol (New England Nuclear) was used as a solubilizer, 10 ml of Dimilum (Packard) was added and radioactivity was determined in a Packard Liquid Scintillation Counter.

[b] Protein was determined as previously described by Bradford (*12*); the values given are the means of four determinations.

[c] Amount of precursor incorporated was calculated from the specific activity of the precursor and the dpm/mg of protein. ND = not done.

Using SDS-polyacrylamide gel electrophoresis, it was shown that glucosamine-^{3}H was incorporated into five or more major glycoprotein bands (bands 1, 2, 3, 4 and 5) and about four minor ones (Fig. 2). The minor bands may have arisen as a result of breakdown of some of the major glycoprotein molecules. The major ones probably represent distinct glycoprotein molecular species associated with the parasite membrane. The glycoproteins of the major bands have approximate relative molecular masses in the ranges of 56 000–81 000 for bands 1 and 2; 98 000–108 000 for 3 and 4; and > 160 000 for band 5. The total absence of peaks in the case of the nonparasitized erythrocytes is further evidence that there is no synthesis of membrane glycoprotein or incorporation of the labelled glucosamine in any other form into the membrane protein of the outdated human erythrocytes used in this study. The significant inhibition of the incorporation of glucosamine-^{3}H into glycoprotein by tunicamycin, a compound known to inhibit glycosylation of protein (*8*), is further evidence that

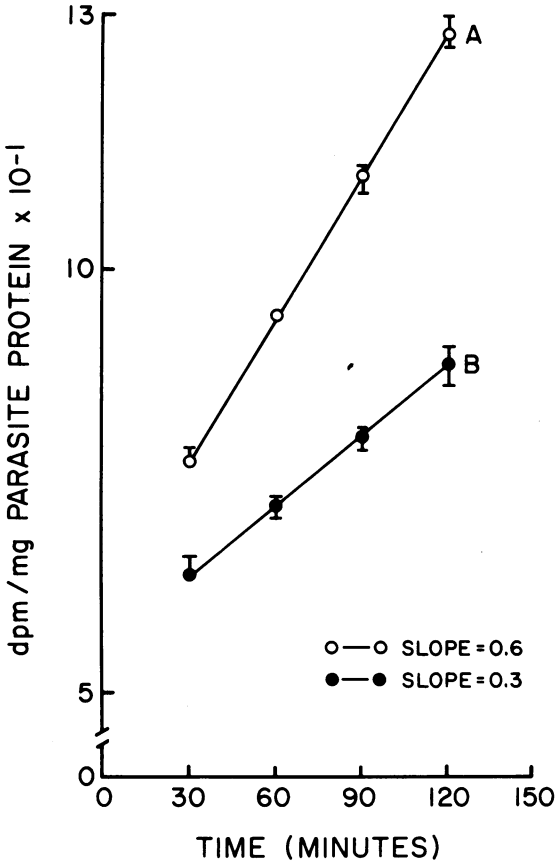

Fig. 1. Rate of incorporation of glucosamine-^{3}H into membrane glycoprotein of *Plasmodium falciparum*. "A" population (open circles) consisted of 81% mature trophozoites and schizont stages and 19% rings; "B" population (black circles) consisted of 90% rings and 10% trophozoites.

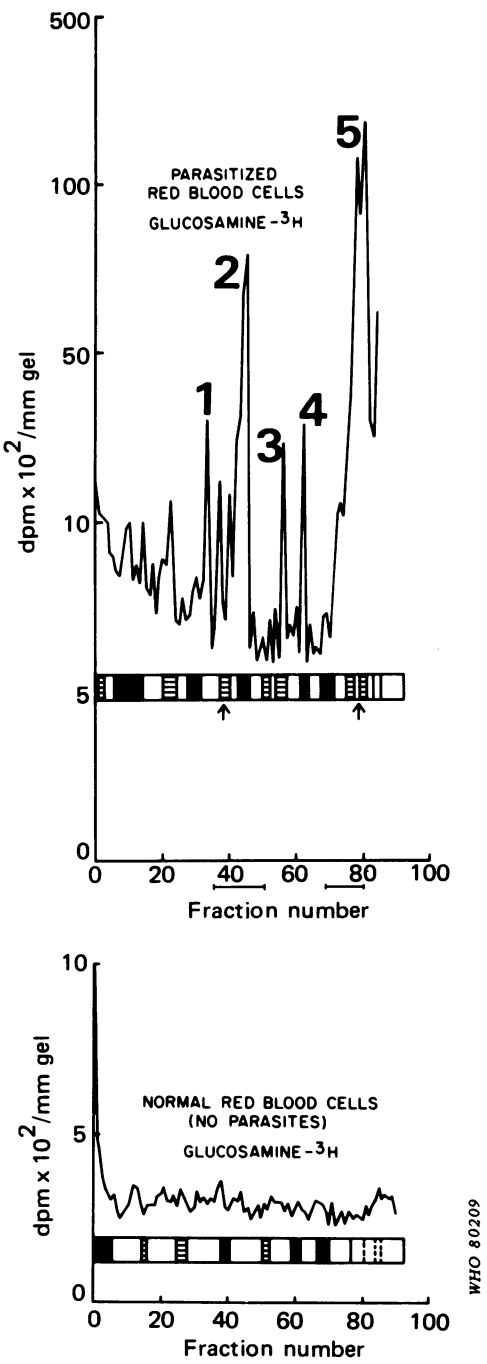

Fig. 2. SDS-polyacrylamide gel electrophoresis of labelled membrane glycoproteins of *Plasmodium falciparum* and of unparasitized erythrocytes.

the precursor was incorporated into glycoprotein as a carbohydrate and not as an amino acid. Conversion of glucosamine to amino acid is limited in *P. falciparum*, which probably lacks a complete Krebs cycle. It also demonstrates the important role of this precursor in parasite glycoprotein synthesis. When 0.01 μg/ml of tunicamycin was added to the incubation medium, incorporation was inhibited by 30% while 1.0 μg/ml produced an inhibition of greater than 50%. A report on the effects of tunicamycin and other agents on the synthesis of membrane glycoprotein in malaria parasites is presented elsewhere (*9*).

This study thus reports the successful labelling of membrane glycoprotein in cultivated *P. falciparum* using small amounts of radioactivity. This method can

be used to investigate the synthesis, turnover, and function of glycoproteins in all parts of the malarial parasite. The thick cell coat covering the malaria parasite at the merozoite stage contains glycoprotein. This coat mediates the attachment of the merozoite to an erythrocyte during invasion, and has been shown by Miller et al. (*10*) to be associated with parasite antigen. Therefore, studies such as this may lead not only to a better understanding of the mechanisms involved in the invasion of erythrocytes by parasites, but they may also help to show whether surface glycoproteins have any role in determining parasite antigenicity, as has been demonstrated for *Trypanosoma brucei* and *T. congolense (11)*.

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RÉSUMÉ

MARQUAGE DES GLYCOPROTÉINES DE LA MEMBRANE DE *PLASMODIUM FALCIPARUM* PROVENANT DE CULTURES

Les travaux décrits montrent que les glycoprotéines de la membrane du parasite du paludisme à *Plasmodium falciparum* en culture continue peuvent être marquées métaboliquement au moyen de petites quantités d'un précurseur radioactif de la glycoprotéine tel que la glucosamine marquée. D'autres précurseurs de glycoprotéines, comme le fucose et le mannose, peuvent aussi être utilisés. Les érythrocytes infectés par les divers stades schizogoniques du parasite ont été obtenus en culture continue dans une cloche à bougie selon la méthode de Jensen et Trager. Ils ont été incubés à 37 °C pendant 48 heures en présence de 74 kBq/ml (2,0 μCi/ml) glucosamine-^{3}H, de fucose-^{3}H ou de mannose-^{14}C. Après incubation, les parasites ont été libérés par lyse à la saponine, et la membrane parasitaire a été elle-même obtenue après lyse hypotonique des parasites libérés. Les fragments de membrane préparés ont été dissous dans du Protosol pour détermination de la radioactivité, ou dans du dodécylsulfate de sodium (SDS) en vue de l'électrophorèse SDS selon la méthode de Laemmli. L'emploi du marqueur glucosamine-^{3}H a permis d'obtenir 9 bandes de glycoprotéine, dont 5 bien visibles et 4 de moindre importance. On ne peut encore affirmer que chacune des bandes représente une glycoprotéine particulière ou que certaines sont des dérivées des principales. Le poids moléculaire relatif de ces dernières se situe autour de 56 000–81 000 pour les bandes 1 et 2, 98 000–108 000 pour les bandes 3 et 4 et >160 000 pour la bande n°5. Après incubation d'érythrocytes non infectés de contrôle en présence de marqueur radioactif, on n'a pas constaté d'incorporation dans leur membrane.

Les méthodes utilisées pour cette étude peuvent être appliquées aux recherches sur la synthèse, le renouvellement et la fonction des glycoprotéines des parasites du paludisme. L'épais revêtement cellulaire recouvrant les mérozoïtes contient des glycoprotéines. Ce revêtement, qui permet l'adhésion de ce stade parasitaire à l'érythrocyte au début du processus d'invasion, est associé, comme l'ont montré les travaux de Miller, à l'antigène parasitaire. Les études de ce type peuvent donc conduire non seulement à une meilleure compréhension des mécanismes en jeu dans l'invasion des érythrocytes par le parasite, mais aussi aider à déterminer si les glycoprotéines de surface jouent un rôle dans l'antigénicité du parasite, comme cela a déjà été démontré pour *Trypanosoma brucei* et *T. congolense*.

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